Charophyte Response to Herbicide and Mycoherbicide Applications

A thesis submitted in partial fulfilment

of the requirements for the degree

of

Masters of Science

in Biological Sciences

at

The University of Waikato

by

Chrystal Leigh Kelly

The University of Waikato

2011

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

Abstract

This thesis describes an investigation into the impacts on charophytes of four selected aquatic herbicide and a mycoherbicide products currently used or under development in New Zealand for alien invasive aquatic plant management. Of particular interest was the charophyte response with respect to oospore germination, germling susceptibility and species response.

In New Zealand, charophytes are native submerged aquatic plants which are recognized as beneficial components of lake ecosystems. They form dense meadows on the lake sediment and are rapid colonisers as they are the first plant to recolonise a lake after a disturbance event. Charophytes produce oospores (seeds) which can remain dormant in seed banks until suitable germination conditions are met. New Zealand lake sediments contain a seed bank which is dominated by charophyte oospores. Alien invasive plants severely impact charophytes by rapidly forming tall, dense monospecific stands which can displace and completely replace the native vegetation.

Chemical weed control in New Zealand is limited to two aquatic herbicides, diquat and endothall, which are currently registered for use on submerged aquatic weeds in lakes and waterways. Fluridone, which is widely used in the USA, is not currently registered for commercial use in New Zealand but has been used in several New Zealand studies. More recently, the development of a mycoherbicide, an inundative biological control, using a formulated naturally occurring aquatic fungus has been trialed in the USA and New Zealand. The effects of these four products on charophytes were investigated in this study. Chelated copper was

included as a control, as it is known to control algae, including charophytes overseas.

For this research, lake sediment was collected from three New Zealand lakes and combined to give one mixed seed bank material of known oospore density and composition. There were two types of charophyte experiments; germination experiments and germling experiments. Two germination experiments examined different scenarios for herbicide treatments under controlled temperature and light conditions. Oospores were either retained in sediment or directly exposed to treatment. Two germling experiments examined germling susceptibility to herbicide treatments under outdoor conditions as well as controlled temperature and light conditions. The second germling experiment included a known charophyte control treatment (chelated copper compound) and a known target invasive plant (*Lagarosiphon major*) control treatment for the herbicides. Herbicide treatment doses started at the maximum label rate and decreased in concentration across a dilution series.

Results from this research indicate that oospore germination was not negatively impacted by any of the herbicide treatments or doses although some species-specific sensitivity was evident. However, further research into species sensitivity is required to ascertain if the sensitivity was due to herbicidal effects or a combination of naturally occurring factors and what the implications of sensitivity are for weed management. For the germling experiments no negative effects were observed for the duration of the study. These results have positive implications for field application of herbicides, indicating that the younger charophyte growth

stages (oospores and germlings) were unaffected by the type of herbicide used at any potential field application rate.

Acknowledgements

There are many people I would like to thank for their contributions to this project. Firstly, I would like to thank my supervisors Professor David Hamilton (University of Waikato), Mary de Winton (NIWA) and Dr Deborah Hofstra (NIWA). Thank you for all your guidance, continual support and always having the time to listen, give advice and lend a hand whenever it was needed.

A big thank you to the Aquatic Plants Group (NIWA) for their continual support through out my research project. To Dr John Clayton and Rohan Wells, thank you for all your support, knowledge and contribution towards my experimental design and herbicide application techniques and procedures.

I would also like to thank Aleki Taumoepeau for his help with the collection of lake sediment from the Rotorua and Hamilton lakes. Dr Fleur Matheson for her knowledge and help with light conversion calculations. Jim Patmore for building the aeration tables. Joshua de Villiers for his help with experimental set-up in the outdoor tanks and Mike Martin for the use of his glassware for the duration of my research project.

Special thanks to the National Institute of Water and Atmospheric Research Ltd (NIWA) for funding my research and providing me with a Study Award. Thank you for giving me the opportunity to undertake my MSc research with you, to learn so much and to have thoroughly enjoyed the experience.

Special thanks also goes to the Biological Sciences Department at the University of Waikato for their financial assistance (funding) towards my participation at the Aquatic Plant Management Society 50th annual meeting in Bonita Springs, Florida, USA. It was a great first time experience presenting an oral presentation at a conference and meeting leading scientists in aquatic plant management.

Special thanks go to Neil Cox, AgResearch Ltd, for all his knowledge and help with the statistical analysis of my data.

Thanks to Dr Deborah Hofstra, Mary de Winton, Professor David Hamilton, Sarah Kelly, Jason Kelly and Ian Kelly for all the hours spent reading my draft thesis and valuable contribution of proofreading.

Last but not least I would like to thank my family for their continual love, support and encouragement. Thanks to Dad (Ian) for all the hours spent filing edges of containers and making a drainage table for one of my experiments.

Preface

This thesis begins with a Literature Review (Chapter 1), which describes aquatic plants, specifically New Zealand native and adventive submerged plants. In Chapter 1 there is also a review of invasive weed control in New Zealand, in particular the chemical and biological control methods. The objectives and hypotheses for the research described in the following chapters of this thesis are outlined at the end of Chapter 1.

Chapter 2 describes the materials and methods used for lake sediment collection, sub-sampling and mixing ratio determination for the mixed seed bank material. It also contains the methods used for the preparation and inoculation of mycoherbicide liquid cultures and the viability tests performed on the liquid cultures to determine which was best for use in each of the experiments. Chapter 2 ends with the material and methods used for each of the four experiments.

Chapters 3 and 4 describe the main findings from the two germination experiments (Experiments 1 and 2 respectively). Chapters 5 and 6 describe the main findings from the two germling experiments (Experiments 3 and 4 respectively). Chapter 7 provides a conclusion, including a summary of the results and possible areas for future research. References follow Chapter 7.

Appendix 1 contains the dilution tables for germination (Experiments 1 and 2) and germling (Experiment 4) experiments. Appendix 2 contains the full mycoherbicide culture viability results for each of the experiments and Appendix

3 concludes with the results from re-isolation of the mycoherbicide agent from the *L. major* treated jar in the second germling experiment.

Table of Contents

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Appendices

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Appendix 3

List of Tables

Chapter 2

Chapter 3

Appendix 1

Appendix 2

Appendix 3

Chapter 1

1. Introduction

This chapter provides background for freshwater plants with a focus on New Zealand natives and adventives. It also describes invasive alien weed control and the two control methods used in this research. The chapter concludes with the research objectives and hypotheses.

1.1. Freshwater Aquatic plants

Aquatic plants are generally concentrated within the littoral zone of lakes and rivers. The littoral zone is defined as the shallow water region with a water depth gradient where light can penetrate to the bottom sediment resulting in a zone of diverse physical conditions ideal for a variety of plant life forms (de Winton & Schwarz, 2004).

The littoral zone may contains three groups of plants; emergent, floating-leaved and submerged plants (Figure 1) (de Winton & Schwarz, 2004; Madsen, 2009b). Emergent plants have their roots and lower stems in the water while their upper stems and leaves extend above the water. Emergent plants are found around the edges of lakes to depths of 2 m in some cases. Bulrush, cattail, sedges and Manchurian wild rice are examples of emergent plants (de Winton & Schwarz, 2004; Madsen, 2009b).

Floating-leaved plants can be broken into two groups; rooted and free-floating. The rooted floating-leaved plants remain anchored in the sediment while their leaves float on the waters surface. Some examples of these include *Potamogeton cheesmanii* (floating-leaved pondweed) and *Nymphaceae* (water lily, spatterdock). Free-floating plants float on the water surface with unanchored roots. Some examples of these include *Lemna* (duckweed), *Azolla* (azolla), *Eichhornia crassipes* (water hyacinth) and *Pistia stratiotes* (water lettuce) (de Winton & Schwarz, 2004; Madsen, 2009b).

Submerged plants are the most abundant plant form in the littoral zone and are found commonly in the deeper depths. They are rooted in the sediment and are permanently under water. The submerged plants are divided into two groups; the low-growing species and the tall-growing species. The low-growing species are plant species that only grow to heights of 0.2 m, some examples include *Glossostigma*, *Lilaeopsis*, *Elatine* and *Isoetes* (quillworts) (de Winton & Schwarz, 2004). The tall-growing species are divided into two groups. The first group, the vascular angiosperms, can grow at depths of 10 m. Examples of these include the natives *Potamogeton ochreatus* and *Myriophyllum triphyllum*, and the introduced oxygen weeds, e.g., *Elodea canadensis* (Canadian pondweed, oxygen weed), *Lagarosiphon major* (oxygen weed), *Egeria densa* (oxygen weed), *Hydrilla verticillata* (Hydrilla) and *Vallisneria* (eel grass) (de Winton & Schwarz, 2004; Madsen, 2009b). The second group of submerged plants are the non-vascular charophytes, for example *Chara* and *Nitella*, which grow at greater depths than the vascular submerged plants (de Winton & Schwarz, 2004).

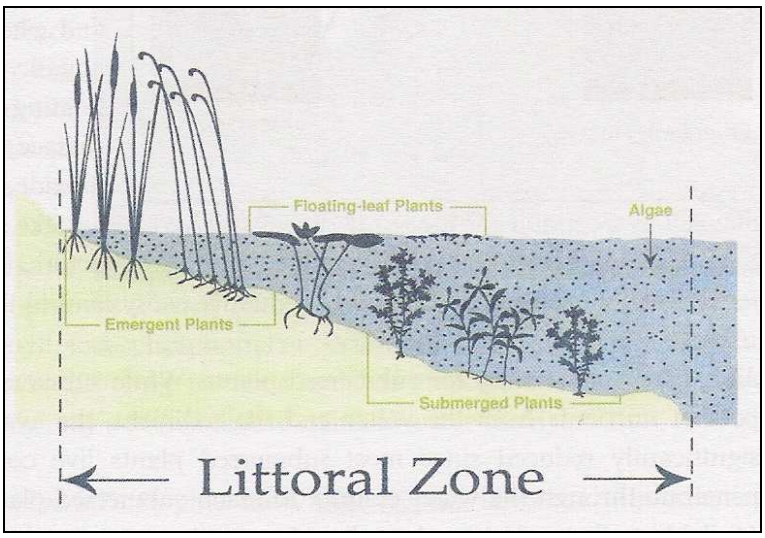


Figure 1: **Zonation of emergent, floating-leaved and submerged plants within the Littoral zone of lakes and rivers (Madsen, 2009)**

Aquatic plants are a valuable component of lake biodiversity (Champion et al., 2002; Madsen, 2009b). Submerged plants in particular play a vital role in creating structurally complex habitats for other aquatic organisms as well as acting as a buffer to surface waves, which reduces water movement and sediment re-suspension (Clayton & Champion, 2003; Duarte, 2000; Santos et al., 2011). Submerged plants remove nutrients from the water column and sediment, and oxygenate the sediment surface layer which helps to reduce nutrient release to the overlaying sediments. However, some submerged plants, generally those that form dense monospecific stands, may also cause localised deoxygenation of the water column (Clayton & Champion, 2003; Duarte, 2000; Santos et al., 2011).

1.2. New Zealand Aquatic Plants

Aquatic plants can be divided into two groups, native and adventive, on the basis of their origin relative to what is present naturally. The term ‘adventive’ means not native. There are several other terms to describe plant species that have spread beyond their natural range which include introduced, alien and exotic. Not all adventive plant species are invasive. Invasive plants refer to plant species whose traits often negatively impact the environment they are in by causing significant changes to the composition (i.e., out-compete natives), structure and ecosystems processes (Closs et al., 2004; Santos et al., 2011). Not all alien species have invasive properties while some native species have invasive properties their environment. In New Zealand the submerged plants that form dense monospecific stands are generally adventive invasive species.

1.2.1. Native Submerged Aquatic Plants - Charophytes

Charophytes, from the Characeae family, form one of New Zealand’s prominent native submerged freshwater plant communities and one of the preferred vegetation types in many waterways. Other native submerged plant communities include Bryophytes, water milfoils (*M. triphyllum and M. propinquum*), pond weeds (*P. ochreatus and P. cheesmanii*), *Isoetes* spp., *Lilaeopsis* spp. and water buttercup (*Ranunculus* spp.) (Coffey & Clayton, 1988).

New Zealand only has four recognised Characeae genera; *Chara*, *Lamprothamnium, Nitella* and *Tolypella* (de Winton et al., 2007; Wood & Mason, 1977). Charophytes are a type of distinctive macroalgae (de Winton et al., 2007; de Winton & Schwarz, 2004) with stems of long single cells alternating with short

nodes where branchlets form. Charophytes produce sexual propagules in the form of spirally ridged oospores, which are analogous to seeds, that vary in size, shape, colour and ornamentation of the outside wall (e.g. sinistral spiral markings, termed striae) (de Winton et al., 2004; de Winton et al., 2007; Mason, 1975) (Figure 2).

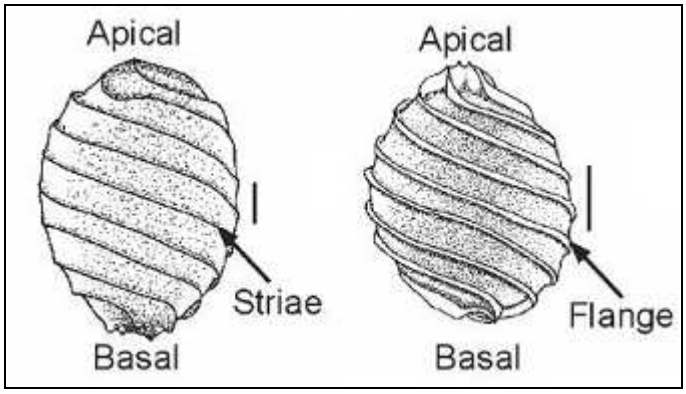


Figure 2: **Morphological features of *Chara* (left) and *Nitella* (right) oospore species. Scale bars = 100 µm (de Winton et al., 2007)**

New Zealand charophyte species are distinguishable from each other by the number of cells comprising the ultimate branchlet and shape of the smaller end cell. The two common charophyte genera found in freshwater are *Chara* and *Nitella* species (Figure 3). *Chara* species have simple unforked branchlets and depending on the species can have corticated stems and branchlets which give them a striped appearance. The *Nitella* plants have forked or repeatedly divided branchlets (Coffey & Clayton, 1988; de Winton et al., 2007; Dugdale et al., 2001; Mason, 1975).

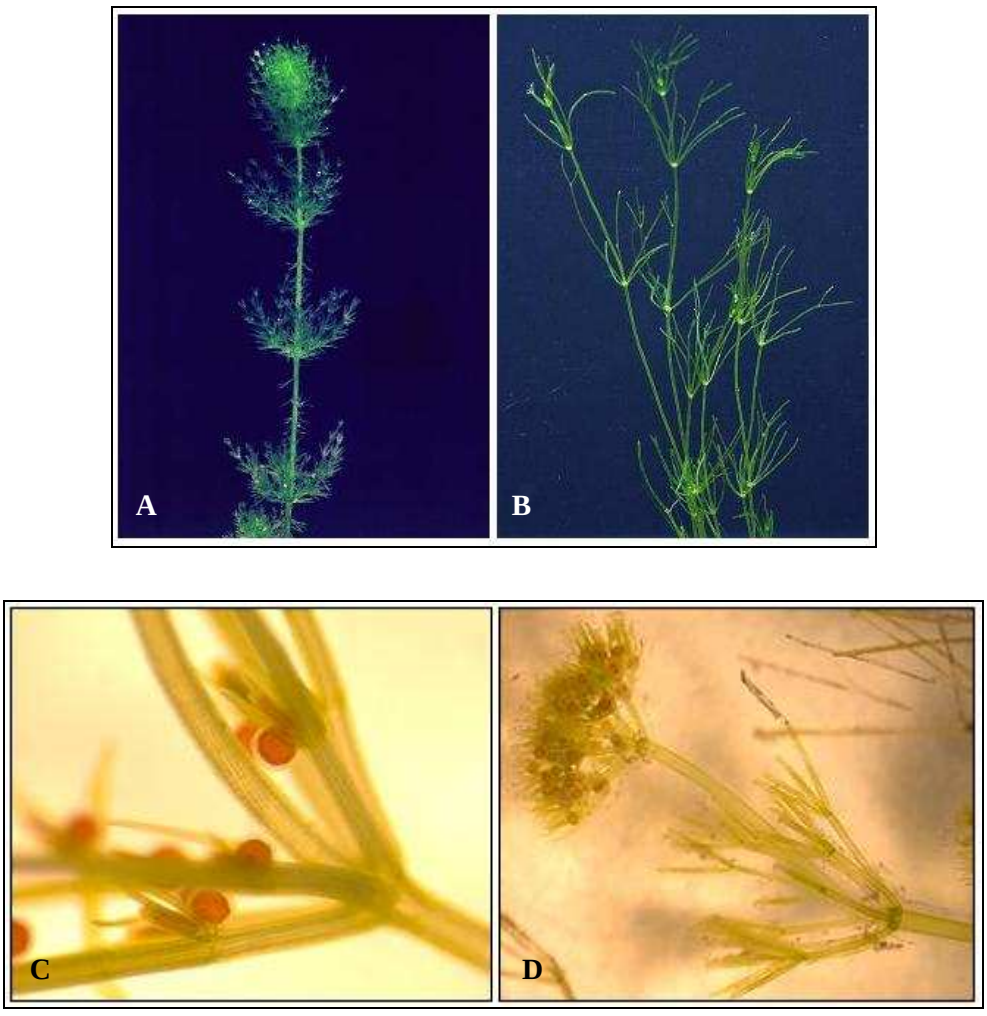


Figure 3: **Photograph illustration of a *Chara* (A and C) and *Nitella* (B and D) species. (A) *Chara fibrosa*, (B) *Nitella leonhardii*, (C) *Chara globularis* and (D) *Nitella pseudoflabellata* (photographs A and B by Aquatic Plants Group, NIWA; photographs C and D by Author)**

Oospore cell walls are thick and multilayered and are formed by the deposition of layers by the developing zygote (oospore) and the surrounding cells (ensheathing cells) (Figure 4). The oospore deposits three layers and the ensheathing cells deposits four layers. The oospore deposits layers on its inside wall while the ensheathing cell deposits layers on its inside wall that is against the oospore, thus creating the oospores thick multilayered cell wall. The oospore-deposited layers consist of a thin electron-dense layer (amorphous layer), a helicoidal microfibril

layer and a non-helicoidal microfibril layer while the enshealthing cell-deposited layers consist of a crystine layer, a pigmented helicoidal microfibril layer, an ornamentation layer and a calcified layer (Leitch, 1989). Once the oospore is fully formed the ensheathing cells break down leaving the oospore behind (Figure 5). The thickened walls are considered to protect the oospores against desiccation and grazing (Casanova, 1991, 1997; Leitch, 1989).

Figure 4: Oospores inside their ensheathing cells (photograph by Author)

Chara oospores are generally larger in size, compared to the *Nitella* species (Figure 5), with a terete (circular) shape in apical view. The *Nitella* oospores are smaller (Figure 5) and non-terete in apical view compared to the *Chara* species (Coffey & Clayton, 1988; de Winton et al., 2007; Dugdale et al., 2001; Mason, 1975)

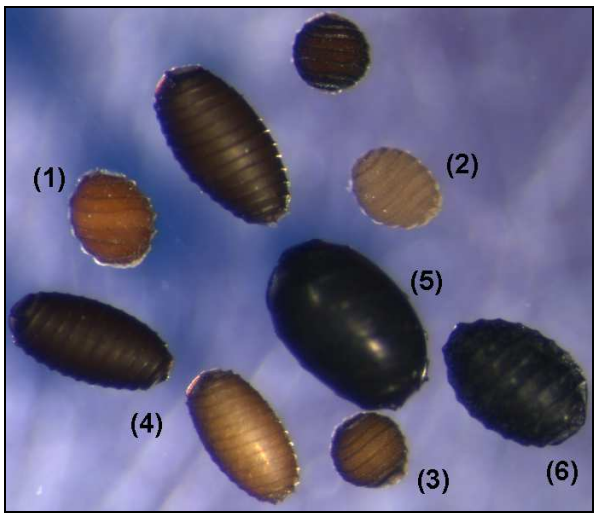


Figure 5: **Selection of *Nitella* and *Chara* oospore species; (1) *N. pseudoflabellata,* (2) *N. hyalina,* (3) *N. leonhardii,* (4) *C. globularis,* (5) *C. australis* and (6) *C. fibrosa* (photograph by Author)**

Charophytes are able to form dense meadows (Figure 6) over a very wide depth range from the shallow littoral zone to 40 m in clear water deep glacial lakes in the South Island (Coffey & Clayton, 1988). Charophyte meadows grow to heights of 2-3 cm in shallow water and over 2 m in deep water (Coffey & Clayton, 1988; de Winton & Schwarz, 2004; Schwarz et al., 2002). One of the reasons thought to contribute to the height change with depth is that charophytes are able to tolerate low light levels and a response to low light levels is that the charophyte cell size increases. Therefore, less light results in larger charophyte plants (Brown, 1975; Kufel & Kufel, 2002). Charophytes have shown a species-specific depth zonation where *C. fibrosa*, *N. hyalina* and *C. globularis* can be found at median depths of 4-5 m, *N. hyalina* and *N. leonhardii* at depths of 5-9 m and *N. pseudoflabellata*, *C. corallina* and *N. hookeri* at depths of 9, 10 and 12 m respectively. This species zonation is thought to be related to the availability of light (Coops, 2002; de Winton & Schwarz, 2004; Schwarz et al., 2002).

Figure 6: **Charophyte meadows in Lake Waikaremoana (photograph by John Clayton, NIWA)**

Charophytes are a beneficial component of lake ecosystems because they are usually the first aquatic plant species to re-establish after a disturbance event and are known as rapid colonisers (de Winton et al., 2000; Van den Berg et al., 1998; van Donk & van de Bund, 2002). Charophytes have two modes of colonisation; vegetative means (bulbils and vegetative propagules) and germination of oospores (diploid zygotes) (Figure 7). Specialised vegetative propagules form during summer described by Starling *et al.* (1974) as consisting of a terminal non-vacuolated internodal cell containing a single whorl of branches and an apical cell (Figure 7).

Figure 7: **Types of charophyte colonisation; (A) bulbil, (B) specialised vegetative propagules and (C) oospores (photographs by Author)**

In New Zealand, lake sediments generally contain a 'seed bank' which is dominated by charophyte oospores (de Winton et al., 2004) from which rapid colonisation occurs. All genera of charophytes primarily reproduce sexually and can have monoecious and dioecious breeding systems so the female fruiting bodies (oogonia) and the male fruiting bodies (antheridia) may either develop in close association of each other, from the nodes of branches (Figure 8) (monoecious) or on separate plants (dioecious). Fertilisation occurs underwater, unlike many of the vascular plants which still rely on aerial fertilisation. After fertilisation, the zygote (also termed oospore) wall thickens (Bold & Wynne, 1978; Smith, 1950). Once the oospores mature they fall off the branchlets into the sediment (Haas, 1994; Kalin & Smith, 2007).

Figure 8: **The male (1) and female (2) fruiting bodies of a *Chara* (A) and *Nitella* (B) species (photograph by Author)**

Oospores are known to have dormancy and longevity characteristics which allow them to remain viable in the lake sediment for many years and to only germinate in large numbers when conditions become favourable. These characteristics allow charophytes to be persistent and rapid colonisers (Bonis & Grillas, 2002; de Winton et al., 2004; de Winton & Clayton, 1996; de Winton et al., 2000; Dugdale et al., 2001; Wade, 1990). Seed banks are important as the oospore composition preserved within them can predict future vegetation composition as well as act as indicators of past condition (Casanova & Brock, 1990; de Winton et al., 2007; Haas, 1994). There are numerous factors that have been examined as possibly influencing oospore germination in lake sediment. These include light, temperature, redox conditions, and oospore size, age and burial depth (de Winton et al., 2000; Dugdale et al., 2001; Kalin & Smith, 2007). The maximum burial depth limit for *Chara* oospores to germinate in New Zealand is 50–100+ mm while *Nitella* have a burial depth limit of <25 mm (Dugdale et al., 2001).

When oospores germinate the initial primary protonema that emerges from the oospore is colourless and only becomes pigmented after cell division to form intermodal cells with nodal cell ends at both ends (Bold & Wynne, 1978; Smith, 1950). Early germling survival is dependent on the starch reserves accumulated in the oospores (de Winton et al., 2004) until the germling becomes pigmented, whereby the germling then moves from a reliance on oospore starch reserves to photosynthetic energy sources (Figure 9).

Figure 9: **Germinating *C. globularis* with the colourless basal cell of the protonema and slightly pigmented secondary cells (photograph by Author)**

Charophyte meadows have the ability to bind sediments which results in a reduction in the water turbidity above the charophyte meadow (Clayton & Tanner, 1988; de Winton et al., 2004; Van den Berg et al., 1998; Van den Berg et al., 1999; van Donk & van de Bund, 2002). Charophyte meadows are also known to act as nutrient sinks. According to Kufel and Kufel (2002), charophyte meadows are able to incorporate the nutrients from the sediment and water column into their biomass as well as enhance sedimentation which prevents nutrients being released from the surficial sediment. Dense unbroken charophyte meadows have been known to inhibit alien weed colonisation and spread as observed in Hamilton

Lake (Lake Rotoroa) (Clayton & Tanner, 1988). Charophytes are, however, severely impacted by increased nutrient loads into lakes causing eutrophication. As the water becomes more turbid the charophytes are restricted to shallow water and can only form small dense mats which are then exposed to wave action. Invasive alien plant species are able to out-compete the charophytes as they adapt better to poor water conditions by forming dense canopies near the water surface (Blindow, 1992; de Winton et al., 2004).

1.2.2. Adventive Submerged Aquatic Plants – Alien Invasive Plants

New Zealand lakes and waterways have been subjected to a number of rapid spectacular invasions by alien plant species (Howard-Williams et al., 1987) since the 1840's when organised settlement began (Mason, 1975). Most of these alien species thrive under New Zealand's conditions and result in domination and displacement of the native vegetation (Brown, 1975; Howard-Williams et al., 1987). The most problematic submerged aquatic plants in New Zealand belong to the Hydrocharitaceae family (namely *Elodea canadensis* Michx., *Lagarosiphon major* (Ridley) Moss ex Wager and *Egeria densa* Planch.) and the Ceratophyllaceae family (*Ceratophyllum demersum* L.) (Champion et al., 2002) (Figure 10).

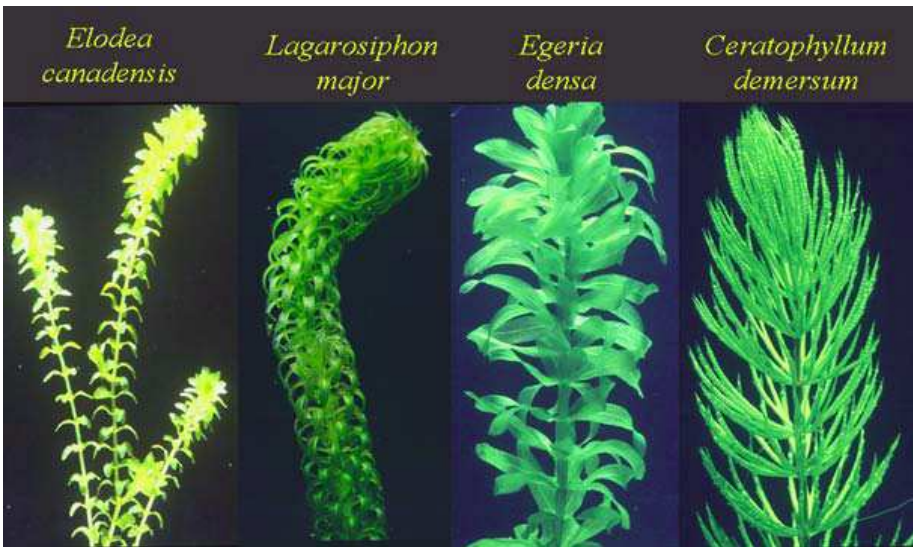


Figure 10: Problematic submerged weeds (increasing weed impact from left to right) (Clayton & Champion, 2003)

E. canadensis is native to North America and was one of the earliest introduced species to become established in New Zealand. It is thought to have been imported into Christchurch with shipments of fish ova in 1868 and deliberately released in efforts to naturalise trout in New Zealand. The first recorded sighting in the natural environment was in 1870 in the Avon River (Howard-Williams et al., 1987). *E. densa* is native to South America and was first recorded in 1946 in the lower Waikato River (de Winton et al., 2009; Hofstra & Champion, 2006d). *L. major* is native to Southern Africa and was first recorded 1950 in the Hutt Valley, Wellington, North Island (de Winton et al., 2009; Hofstra & Champion, 2006e). These oxygen weeds were thought to have been imported for their ornamental and habitat enhancement value. By 1982 they were wide spread through the aquarium trade (Howard-Williams et al., 1987). *C. demersum* (hornwort) is native to many temperate and tropical regions (Hofstra & Champion, 2006c). It was first recorded in drains near Napier and Hastings in 1961 and was likely an aquarium escapee. In 1963 it was found in the Waikato River (Howard-Williams et al., 1987; Mason,

1975) and subsequently spread throughout the North Island and recently recorded (from 2000) in several locations in the South Island (de Winton et al., 2009).

There are no native representatives of the Hydrocharitaceae or Ceratophyllaceae family in New Zealand (Mason, 1975). The species introduced to New Zealand are generally fast growing tall plants which form dense monospecific stands that are often surface reaching which create a shading effect on smaller native species (Closs et al., 2004; Coffey & Clayton, 1988). They are able to grow to depths of 6-10 m (*L. major* and *E. densa* respectively). However, hornwort is able to impact even greater depths (Champion et al., 2002) as was recorded in Lake Tarawera where it was recorded at depths of 15.5 m (Wells et al., 1997) (Figure 11). In New Zealand most of these species are dioecious and only represented by either the male or female plant. Therefore, they can only propagate vegetatively. Fragments of stems containing a viable bud at the stem nodes can form adventitious roots for anchorage in the sediment and new apical shoots. Hornwort, which is monoecious, is known to reproduce through seeds in other countries. However, no viable seed has been recorded in New Zealand. This is possibly due to unfavourable environmental conditions that prevent sexual reproduction and therefore fragmentation of stems is the only means of reproduction (Closs et al., 2004; Coffey & Clayton, 1988; Hofstra & Champion, 2006c; Howard-Williams et al., 1987; Mason, 1975).

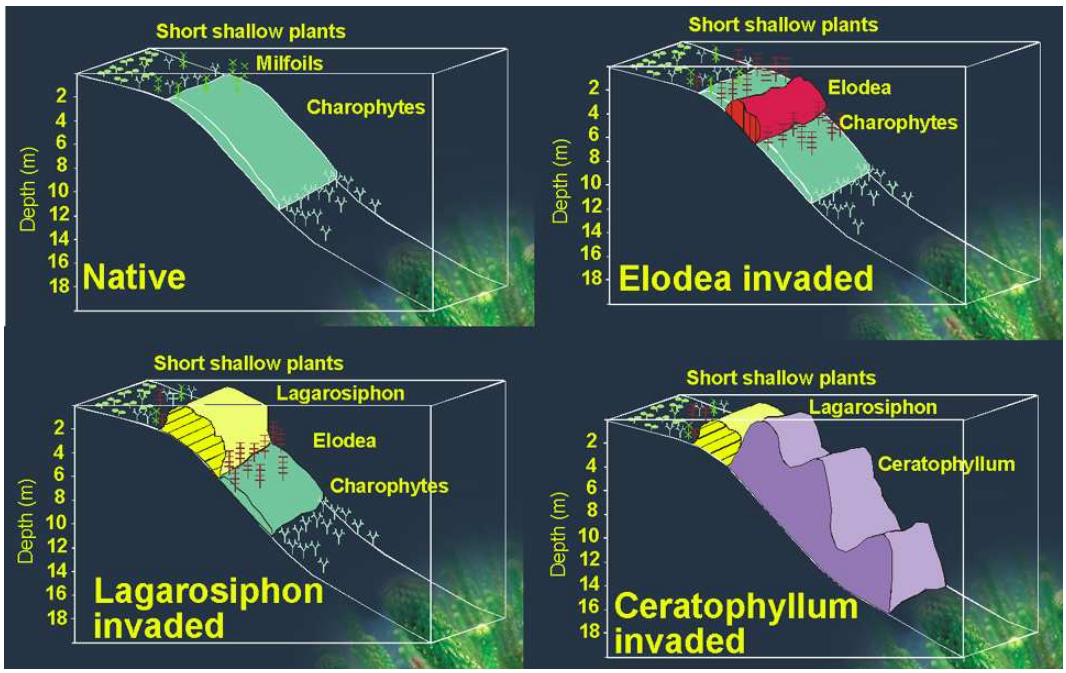


Figure 11: **Diagrammatic representation of the invasion sequence of Lake Tarawera (native vegetation pre- 1900s, *Elodea* invasion 1930s, *lagarosiphon* invasion 1960s, *Ceratophyllum* invasion 1990s) (Clayton & Champion, 2003)**

The rapid spread of these tall-growing invasive weed species has detrimental effects on native plant species and biota. Since these alien plants are able to form large dense monospecific stands which reduce light penetration into the water column, increase organic matter in the sediment and reduce oxygen levels in the water column (Closs et al., 2004), the biota living in and on the sediment are severely impacted by the resulting changes in their natural habitat (Champion et al., 2002). These alien plants are able to completely replace native plants (Champion et al., 2002; de Winton & Clayton, 1996) or displace them to deeper areas where light is limited or to shallower areas where they are more exposed to wave action and desiccation (Closs et al., 2004). Displacement of native plants has detrimental impacts on native seed banks, thus limiting the emergence, growth and reproduction of charophytes (Bonis & Grillas, 2002; de Winton & Clayton, 1996). Biota adapted to living in the native beds are also likely be impacted by

their displacement. However, some alien plant species in moderate abundance provide food for birds and refuge and habitat for invertebrates and epiphyton (Champion et al., 2002).

The rapid growth and spread of alien plant species not only impacts native biota within the lake but can also cause economic, recreational and aesthetic problems. Dense stands of alien species, especially hornwort, may interfere with hydroelectric generation when floating mats of it clogs up the water intakes for turbines. This has resulted in hydroelectric dams shutting down temporarily, losing several millions of dollars in power generation and necessitating costly repairs (Champion et al., 2002; Closs et al., 2004). The accumulation of alien weeds devalues waterfront properties and causes declines in tourism due to unsightly views and smells when the weeds are washed ashore and decay. Surface-reaching weeds affect recreational activities such as swimming, boating, water skiing and fishing as the weeds can tangle up the swimmers, boat propellers, water skiers and fishing lines, causing damage and even death (Champion et al., 2002; Closs et al., 2004). Excessive weeds near shore restrict access of boats to the open water as weeds entangle boat trailers and boats at boat ramps and jetties (Closs et al., 2004).

1.3. Invasive Alien Weed Control

The impact of invasive plants and the management objectives to remove or decrease weed beds of alien species has led to the development of aquatic control technologies in four broad categories; mechanical/physical removal, habitat manipulation, chemical control and biological control (Champion et al., 2002;

Closs et al., 2004). The choice of management options is determined by the weed species and site-specific management factors, for example, type of water body, priority of the site, weed issues, water body uses, environmental constraints and feasibility (Champion et al., 2002; Closs et al., 2004, p. 27.28; Madsen, 2009a). The two aquatic control techniques used in my research involved chemical control, which is the most widely used option in New Zealand for large scale weed control (Champion et al., 2002), and biological control (inundative), which in this case is a fungal plant pathogen formulated into a mycoherbicide (Shearer, 1996b).

1.3.1. Chemical Control Method: Aquatic Herbicides and Algaecide

Aquatic herbicides are used in lakes and waterways with the aim of reducing target plant species. Only registered aquatic herbicides are used as they pose low risks to the aquatic environment and biota as long as they are applied at the rates stated on their labels (Hofstra & Champion, 2006b). Aquatic herbicides contain both active ingredients, i.e. the chemicals that control the target weeds, and inactive (inert) ingredients that can either dilute, improve or make the herbicides easier to mix or apply. Some herbicides use carriers such as oil, water or clay as inert ingredients to improve the mixing and application of the herbicide (Avery, 2003). There are limitations to herbicide use in aquatic environments for successful target plant control. For example, when there is a lot of water movement at the treatment site the herbicides become rapidly diluted after application. This makes it difficult to maintain the required herbicide concentration and contact time (Hofstra & Champion, 2006b). Plant species differ in their susceptibility (ability to be controlled, partially controlled, tolerant or resistant) to herbicides. Therefore selective control of target species can be

achieved through choice of herbicide and contact/exposure time relationships (Glomski et al., 2005; Hofstra et al., 2001). Herbicide applications in New Zealand have shown effective seasonal control of target plants with minimal to no impact on non-target plants including mature charophytes (Clayton & Matheson, 2010; Hofstra & Champion, 2006b). This has lead to the main area of interest for this research, which focuses on the possible impacts of aquatic herbicides on the potentially vulnerable life stages of native charophytes.

Diquat and endothall are the only aquatic herbicides registered for use on submerged invasive plants in standing and flowing water in New Zealand (Hofstra & Champion, 2006a). Fluridone is currently not registered in New Zealand but is widely used throughout the USA for invasive weed management (Siemering et al., 2008) and has also been used in some trials in New Zealand (Hofstra & Clayton, 2001a; Wells et al., 1986). Chelated copper compounds are used in the USA and India specifically to control algae, but has also been used to control aquatic vascular plants (Durborow et al., 2007; Guha, 1995; Leslie, 1990). For this study, three aquatic herbicides (diquat, endothall and fluridone) and one algaecide (chelated copper compound, K-Tea) were chosen for use. Diquat, endothall and fluridone were used in all experiments while the chelated copper compound was only used in the fourth experiment.

Diquat

Diquat (Reglone®, Syngenta) was the first aquatic herbicide registered in New Zealand and has been used since the 1960s. Diquat is formulated as dibromide salts and is a fast-acting contact herbicide which disrupts the electron transport

system in plant photosynthesis and damages cell membranes and cytoplasm, which leads to desiccation or defoliation. It only requires a short contact time to affect the target plant species. However, since diquat is a contact herbicide, any parts of the plant exposed to inadequate herbicide levels or contact time are likely to regenerate. Diquat is considered to be a 'broad spectrum' herbicide that can be used on a wide range of target alien species (*E. canadensis*, *E. densa*, *L. major* and *C. demersum*). However, in a turbid aquatic environment, diquat cations are rapidly bound to negatively charged clay and sediment particles in the water column or on the plant surface, resulting in the rapid deactivation of its herbicidal effects. Diquat is also degraded in the aquatic environment by microbial action. The general exposure requirement for diquat is hours to days and the typical half life of diquat is 0.5 to 7 days (Clayton, 1986; Hofstra et al., 2001; Netherland, 2009; Simsiman et al., 1976).

Diquat has shown effective control of target species in New Zealand (*C. demersum*, *L. major*, *E. densa* and *E. canadensis*) while maintaining, and in several cases enhancing, charophyte vegetation (Clayton, 1986; Clayton & Matheson, 2010; Hofstra et al., 2001; Leonard & Creenland, 1965; Tanner et al., 1990; Wells & Clayton, 1993)

Endothall

Endothall (Aquathol® K, United Phosphorous Inc), a dicarboxylic acid, is the second herbicide registered (in 2004) for aquatic use in New Zealand. Endothall is similar to diquat in that it is a contact herbicide. Endothall is formulated as a dipotassium salt and is known to inhibit protein synthesis and cause disruption of

membranes and respiration, which results in wilting, desiccation and collapse of the treated plants. Unlike diquat, endothall does not bind to charged clay and sediment particles. Plant species have differing sensitivity to endothall, and in New Zealand it has been shown that it has no impact on the target weed species *E. densa*. The general exposure requirement for endothall is hours to days. The typical half life of endothall is 2 to 14+ days but it could be less (4 – 7 days) depending on the temperature of the water and the prevalence of microorganisms that break it down into naturally occurring compounds (Hofstra & Champion, 2008; Hofstra & Clayton, 2001b; Hofstra et al., 2001; Netherland, 2009; Simsiman et al., 1976).

In New Zealand, endothall is potentially able to control several target species (*C. demersum*, *Potamogeton crispus*, *L. major* and *Hydrilla verticillata*) within 22-48 hrs at the maximum label concentration of 5 ppm while other target species (*E. densa* and *E. canadensis*) and non-target species (charophytes) show no susceptibility within the 0.5–5 ppm concentration range (Hofstra et al., 2001; Wells & Clayton, 1993)

Fluridone

Fluridone (Sonar® AS, SePRO) is currently not registered in New Zealand but is widely used throughout the United States in invasive weed management and has been used in New Zealand in several experimental trials (Hofstra & Clayton, 2001a; Wells et al., 1986). Fluridone, 1-methyl-3-phenyl-5-[3-(trifluoromethyl) phenyl], also known as a ‘bleaching herbicide’, is a systemic herbicide that inhibits the formation of carotenoid pigments essential for normal plant growth.

The impact is observed in the new shoot growth which is often white as the chlorophyll is destroyed by sunlight. Fluridone is usually degraded in the environment through photolysis and microbial action. Fluridone is a slow-acting herbicide with the general exposure requirement of 45+ days and a half life of 7–30+ days (Doong et al., 1993; Netherland, 2009; Wells et al., 1986).

In field surveys in the USA, charophytes were reported to be unaffected by fluridone while angiosperms were destroyed (Burkhart & Stross, 1990; Netherland et al., 1997). This resulted in studies on the effect of fluridone on charophyte sporelings (germlings) which showed that the bleaching effect increased with increasing fluridone concentrations (0.01–10 ppm). However, at maximum concentration (10 ppm) oospore germination was unaffected (Burkhart & Stross, 1990). Charophytes have also been observed to germinate and colonise in pots where the target species were reduced after fluridone treatment (Netherland et al., 1997). In New Zealand, fluridone trials on target species (*L. major, E. canadensis, E. densa, H. verticillata, Vallisneria gigantea, P. crispus* and *Salvinia molesta*) resulted in bleaching or pink colouration of new shoots on plant stems. However, this had no overall detrimental effects on the plants' growth as the plants recovered (Hofstra & Clayton, 2001a; Wells et al., 1986)

K-Tea

K-Tea (K-Tea®, SePRO) is a chelated copper compound derived from Copper – Triethanolamine complex and Copper Hydroxide and is commonly used as an algaecide. Copper in a chelated compound does not readily precipitate in the water column, allowing it to remain active for longer. Chelated copper compounds are

fast-acting, with general exposure requirements of a minimum of three hours. Copper is a natural element and does not biodegrade in the water column. However, it remains biologically inactive in the sediment as free copper ions bound to sediment. The typical half life of copper in water is hours to 1+ days as copper binds to particles or chemical ions in the water or sediment. Water alkalinity also affects the half life of copper by making the copper more readily inactive (Morris, 2009; Netherland, 2009)

Chelated copper compounds are known chemical controls for charophyte species (Durborow et al., 2007; Peterson & Lee, 2005) and copper compounds have been used in India to control *Chara* species in Kharif rice fields (Guha, 1995). Copper compound have also been used to control vascular submerged plants, i.e., Komeen. However, copper compounds are usually combined with another herbicide, such as diquat, endothall and fluridone to improve effectiveness (Durborow et al., 2007). For example, when only copper is used, the pathological changes (toxicity) occurred in *Hydrilla verticillata* at 80-100 mg Cu/kg plant tissue, whereas, in combination with diquat it occurred at 30-40 mg Cu/kg plant tissue (Leslie, 1990)

1.3.2. Biological Control Method: Mycoherbicide

Mycoherbicide Concept

The mycoherbicide strategy uses formulated pathogens to control nuisance plants in ways consistent with herbicide technology and equipment (Shearer, 1994). In the USA *Mycoleptodiscus terrestris* (Mt) has been identified as an ideal candidate for inundative biological control (Shearer, 1998), which requires that the Mt be

formulated into a product that has a shelf life and can be applied with conventional spraying equipment (Shearer, 1997). The inundative approach, described by Shearer (1998), varies from the classical biocontrol technique in that a plant pathogen is used to control a target weed in a specific area through predetermined volumes and doses. This allows the control of the target weed within a specific time frame, thus reducing any economic losses. In comparison, the classical biocontrol method involves the release of a host-specific control agent, for example, a beetle (*Agasicles hygrophila*) used to control alligator weed (*Alternanthera philoxeroides*) (Closs et al., 2004), that forms a self-sustaining population in the wild. This population will then vary in density or numbers with the population size of the target species (Shearer, 1996b).

In the 1970s, initial investigations into the used of Mt as an inundative biological control agent started in the USA when Mt was isolated from *Myriophyllum spicatum* in Massachusetts (Shearer, 1994). Throughout the 1980s and early 1990s fungal isolates were studied and evaluated in laboratory, greenhouse and field tests. EcoScience Corporation (Worcester, MA) sought to commercialise an isolate of Mt as a mycoherbicide (Aqua-Fyte) after promising field trial results using liquid inoculum (Shearer & Jackson, 2006). However, the first two field tests (in 1994 and 1996) of Aqua-Fyte were ineffective at reducing aboveground biomass of Eurasian watermilfoil (*Myriophyllum spicatum* L.) and the cause was put down to problems with the fungus and/or formulation of the mycoherbicide (Shearer, 1994, 1996b). Subsequent research on Mt has been carried out by Judy Shearer (USA ERDC – US Army Corp of Engineers Research and Development Center), Mark Jackson (USDA – Agricultural Research Service USA), Mark

Heilman (SePRO corporation, USA) and the Aquatic Plants Group (NIWA, New Zealand).

Mycoleptodiscus terrestris (Gerd.) Ostazeski Species Description

The genus *Mycoleptodiscus* comprises 15 species of fungi from a diverse host range including forage legumes (Ostazeski, 1967), eucalypts (Sutton & Hodges Jr, 1976) and conifers (Bills & Polishook, 1992). Since its original description in the United States (Gerdemann, 1953; Ostazeski, 1967), *M. terrestris* (Mt) appears to have a cosmopolitan distribution and a host range that includes submerged aquatic macrophytes, for example, Eurasian watermilfoil (*Myriophyllum spicatum* L.) and hydrilla (*Hydrilla verticillata* (Lf) Royle) (Shearer, 1997). Mt is a plant pathogen which has been found on asymptomatic or slightly infected plants (i.e. presence of lesions) and when applied in greater amounts (inundative exposure) causes significant symptoms, for example the plants become chlorotic, flaccid and disarticulated (Shearer, 1998).

Mt has been described as having yellowish cream–coloured sporodochia up to 100 μm in size that produce boat-shaped or cylindrical conidia (spores) with rounded tips (Figure 12). The conidia are aseptate or one-septate, bearing two straight (or very rarely curved) filiform appendages at both ends laterally on one side, although an absence of appendages has also been reported (Hofstra et al., 2009). Microsclerotia, formed in liquid cultures, are melanised, compact hyphal aggregates which are spherical or elongated in shape and are known to be highly resistant to desiccation and often serve as the over-wintering structure of the fungus (Shearer & Jackson, 2006; Watanabe et al., 1997).

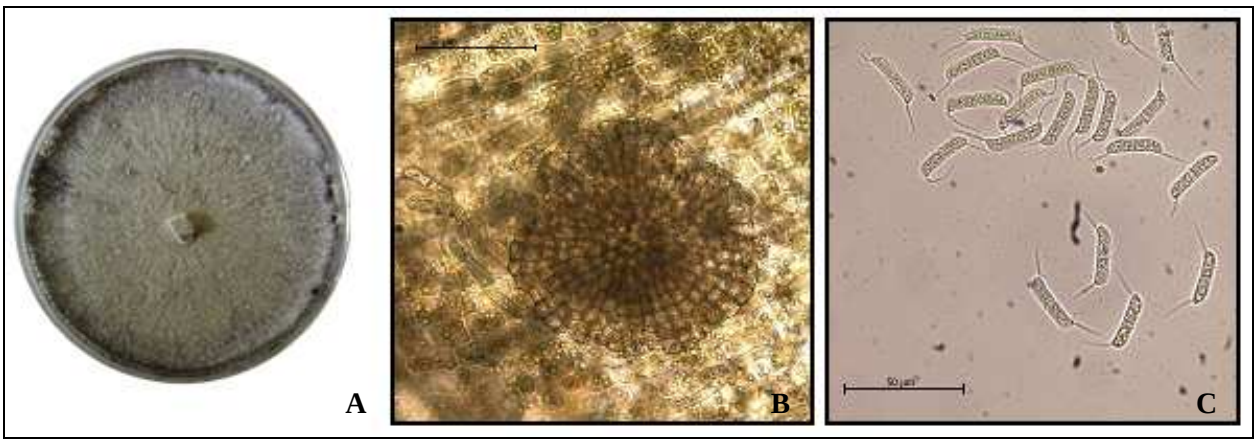


Figure 12: **An *M. terrestris* isolate growing on a potato dextrose agar plate (A), a sporodochia on a plant leaf (B) and Mt spores produced by the sporodochia (C). Scale bars = 50 µm and diameter of plate = 90 mm (photograph A by Deborah Hofstra, NIWA; B and C photograph by Author)**

M. terrestris in New Zealand

During a plant pathogen survey of aquatic macrophytes from lakes and waterways in New Zealand's North Island, a *Mycoleptodiscus*-like fungus was isolated from *Ceratophyllum demersum* in the Waikato River (Hofstra et al., 2009). When the fungal isolate was plated onto standard laboratory media for identification, the isolate produced sclerotia characteristic of the *Mycoleptodiscus* fungus but not the definitive two-celled appendaged spores (Hofstra et al., 2009). In developing techniques for producing *M. terrestris* sclerotia in liquid culture in the USA, certain selective media (i.e. solylus, casamino acids, pharmamedia) enabled sclerotia to consistently germinate sporogenically, providing copious numbers of spores (Shearer, 2007; Shearer & Jackson, 2003, 2006). These techniques developed in the USA were successfully used to culture one of the New Zealand isolates resulting in the positive identification of *M. terrestris* (Hofstra et al., 2009). This and subsequent New Zealand Mt isolates are stored and maintained in NIWA's culture collection in Hamilton, New Zealand.

Currently there is no data on aquatic plant specificity of *M. terrestris* in New Zealand and current research is investigating the host range of *M. terrestris*. In USA, however, *M. terrestris* field and laboratory trials show infection of aquatic plant species such as *Hydrilla verticillata* and Eurasian watermilfoil (Shearer, 1994, 1998). Liquid culture dose rates used in the USA trials are based on the colony forming unit (CFU) count of 1×10^6 CFU mL^{-1} (Shearer, 1996a), where as the dose rates used in the New Zealand trials are based on *M. terrestris* biomass in the liquid cultures.

1.4. Research Objectives and Hypotheses

The overall aim of this Master of Science (MSc) research was to determine how selected aquatic herbicides and a mycoherbicide affect charophyte germination or germling growth. Of particular interest was how charophytes responded in terms of germination success, germling susceptibility and species sensitivity.

Hypotheses were that firstly, charophyte germination would not be affected by herbicide and mycoherbicide treatments at concentrations representing potential field application rates to control invasive species and, secondly, that germling charophytes would not be susceptible to these treatments.

To date, germling and oospore germination has received relatively little study. Therefore this MSc research provides insight into what effects aquatic herbicides and a mycoherbicide have on charophytes' germination, germling susceptibility

and species sensitivity. This is important for aquatic plant management as the research may indicate that, given the same level of target weed control, the use of one product (diquat, endothall, fluridone and mycoherbicide) over another may selectively control alien species but leave charophytes unaffected.

Chapter 2

2. Materials and Methods

This chapter describes the experimental methods used for the collection and preparation of the lake sediment, the processes involved in making, preparing and viability testing mycoherbicide liquid cultures and then concludes with experimental methods used for each germination (Experiment 1 and 2) and germling (Experiment 3 and 4) experiment.

2.1. Lake Sediment (Seed Bank Material)

Lake sediment (seed bank material) was used in each experiment as it contains large reserves of charophyte oospores. The lake sediment was collected from three lakes (Lake Tarawera, Lake Tikitapu and Lake Rotoroa) and a mixed seed bank material was prepared as outlined below.

2.1.1. Collection

Approximately 50–60 L (total volume) of lake sediment containing oospores, hereafter referred to as seed bank material, was collected from Lake Tarawera, Lake Tikitapu (Blue Lake) and Lake Rotoroa (Hamilton Lake). NZ Map Grid reference coordinates and depths of sampling of these lakes are given in Table 1 and Figure 13 shows a map of the lakes. Sediments were collected from these lakes to give a mixed age and species composition of *Nitella* and *Chara* species.

Table 1: Collection sites for lake seed bank material

Collection Site	Grid reference	Depth (m)
Lake Tarawera	2806270E, 6327200N	5-7
Lake Tikitapu	2801700E, 6329590N	5-7
Lake Rotoroa	2710540E, 6375750N	1.5

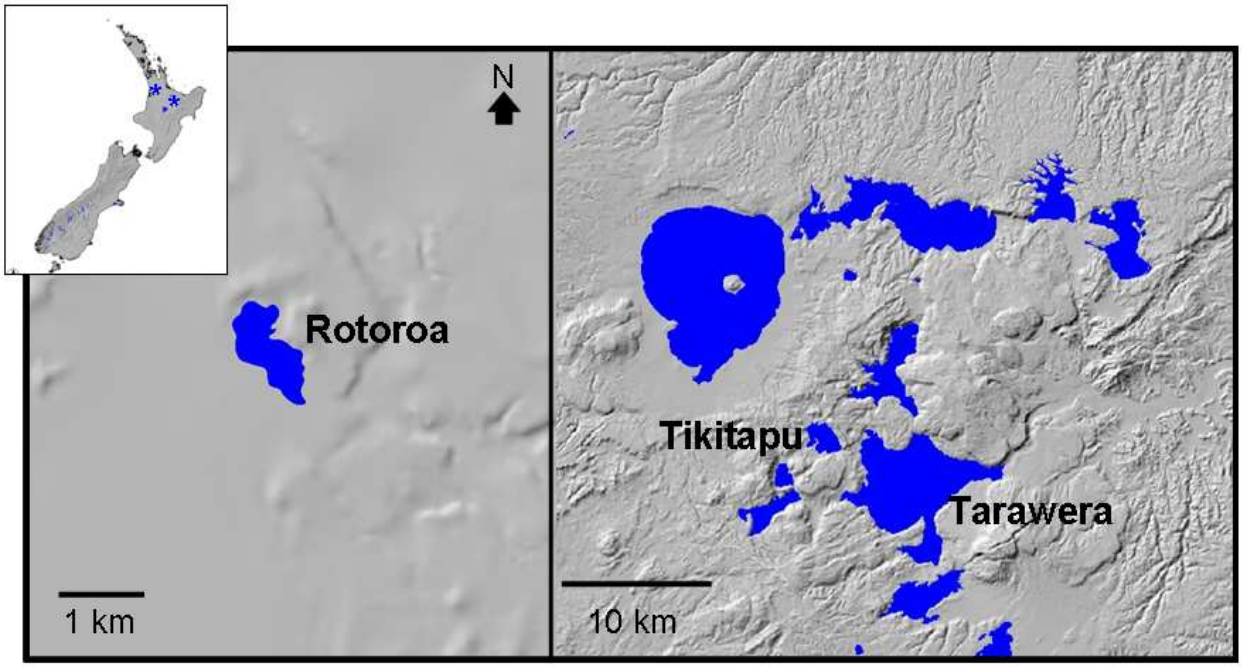


Figure 13: Map showing the location of Lake Rotoroa (Hamilton) and Lake Tarawera and Lake Tikitapu (Rotorua Lakes District) (map supplied by Mary de Winton, NIWA)

2.1.2. Preparation

Each of the lake sediments was sieved separately through a large garden sieve (12 mm mesh) into separate containers to remove any plant matter and debris from the sediment. The sieved sediment from each lake was thoroughly mixed to homogenise the sediment before sub-samples were taken for analysis of oospore composition.

2.1.3. Sub-sampling of Lake Sediments

Sub-samples from each lake were sieved further to estimate the density and composition of oospores. Sub-sample volumes were 10 mL (Lake Tarawera and Lake Tikitapu) or 100 mL (Lake Rotoroa). The larger sub-sample volume for Lake Rotoroa was due to the lower density of oospores present. Sub-sampling was repeated four times from each lake's sediment. Two different mesh sizes (250 µm and 500 µm) were used to sieve the sediment. The 500 µm sieve was placed on top of the 250 µm sieve and the sediment sample was washed into the 500 µm sieve. The contents of the sieves were placed into separate glass petri dishes and examined under a stereo microscope (Leica MZ 9.5, Leica Microsystems, Bio-Strategy Ltd).

The oospores present were identified according to de Winton et al. (2007), counted and recorded (Tables 2-4). The overall oospore concentration in the seed bank material was 24.35 oospores/mL (Lake Tarawera), 21.33 oospores/mL (Lake Tikitapu) and 0.38 oospores/mL (Lake Rotoroa). Lake Tarawera had the most diverse oospore composition while Lake Tikitapu had the least diverse oospore composition.

Table 2: **Oospore composition and concentration for each 10 mL sub-sample taken from the lake sediment collected from Lake Tarawera**

Charophyte species	Sub-sample 1	Sub-sample 2	Sub-sample 3	Sub-sample 4
Chara globularis	245	167	210	191
Chara fibrosa	3	-	1	1
Chara australis	-	-	1	-
Nitella hyalina	25	27	28	13
Nitella pseudoflabellata	9	8	4	5
Nitella leonhardii	10	11	9	7

Table 3: **Oospore composition and concentration for each 10 mL sub-sample taken from the lake sediment collected from Lake Tikitapu**

Charophyte species	Sub-sample 1	Sub-sample 2	Sub-sample 3	Sub-sample 4
Nitella pseudoflabellata	153	165	147	167
Nitella leonhardii	56	55	71	39

Table 4: **Oospore composition and concentration for each 100 mL sub-sample taken from the lake sediment collected from Lake Rotoroa**

Charophyte species	Sub-sample 1	Sub-sample 2	Sub-sample 3	Sub-sample 4
Chara australis	22	25	24	29
Nitella aff. *cristata*	6	15	13	14
Nitella pseudoflabellata	1	1	-	3

2.1.4. Mixing Ratio

Once the oospore composition and concentration were determined for each lake, the three lake sediments were mixed according to two different mixing ratios. Mixing ratio A was used for Experiments 1 and 3 and mixing ratio B was used for Experiments 2 and 4 (Table 5). The second mixing ratio (B) used all the remaining lake sediment not used for mixing ratio (A). The mixing ratios were calculated taking into account that published oospore germination rates from natural sediment range from 5–50%. The lower germination rate of 5% was used as the experimental baseline germination rate (de Winton et al., 2004; de Winton et al., 2000). The aim was to have a mixing ratio that potentially yielded 100 germinating oospores in 120 mL of the mixed lake sediment (Table 5). Once the lake sediments were mixed, they were stored at 2 ºC.

Table 5: **Mixing ratios for Lake Tarawera, Lake Tikitapu and Lake Rotoroa sediments. (A) Experiments 1 and 3. (B) Experiments 2 and 4.**

Lake	Oospore count. in 120 mL sediment	Potential number of germinating oospores in 120 mL sediment*	Mixing Ratio		Potential number of germinating oospore in 120 mL mixed sediment*	
			(A)	(B)	(A)	(B)
Lake Tarawera	2922	146.1	2/5	13/34	58.44	55.86
Lake Tikitapu	2559	127.95	2/5	11/34	51.18	41.39
Lake Rotoroa	45.6	2.28	1/5	10/34	0.456	0.67
The total number of germination oospores expected in 120 mL mixed lake sediment					110.1	97.9

* based on 5% germination

2.2. Mycoherbicide Liquid Cultures

The liquid cultures of mycoherbicide inoculum were prepared as per Shearer and Jackson (2006). The steps involved preparing agar plates, plating *Mycoleptodiscus terrestris* (Mt) for inoculating liquid cultures, preparing and inoculating liquid cultures, culture viability testing and preparing the final mycoherbicide inoculum.

2.2.1. Preparing Agar Plates

Two types of agar were used: WA (water agar) for spore plates and PDA (potato dextrose agar) for culture growth plates and plating Mt isolates. Either 20 g Agar, granulated (Difco Laboratories media) (for WA plates) or 39 g Potato Dextrose Agar (Difco Laboratories media) (for PDA plates) was added to 1 L distilled water in a Schott bottle. The Schott bottle lids were loosened and secured in position with autoclave tape. The Schott bottles were then autoclaved at 121°C for 15 minutes. After autoclaving, the Schott bottles were placed in the laminar flow cabinet to cool and once the bottles were warm to touch, the agar was poured into plastic Petri dishes (LabServ®) and allowed to set.

2.2.2. Plating *Mycoleptodiscus terrestris* (Mt)

The New Zealand Mt isolate used for my research was Mt1 Rjssp. Vials of this Mt isolate were retrieved from cryostorage (Thermo Scientific Revco Ultima Plus, Bio-Strategy Ltd) and thawed. To initiate colony development, Mt was plated onto Potato Dextrose Agar (PDA) in a laminar flow cabinet using sterile tweezers, to form a colony plate (Figure 14). The Mt colony plates were grown for seven days (at 27 ºC) before being used to inoculate liquid culture.

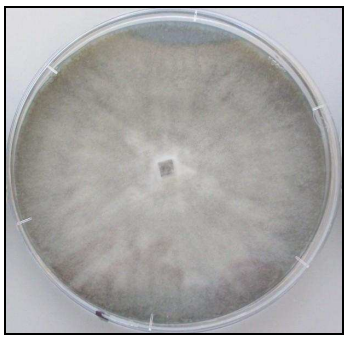


Figure 14: **Example of an Mt colony plate used for one of the experiments. Plate diameter = 90 mm (photograph by Author)**

2.2.3. Preparing Liquid Cultures

Liquid cultures were prepared in 500 mL Erlenmeyer flasks containing 190 mL of media. The liquid media comprised of 20% glucose solution (75 mL), Casamino acid media (Difco Laboratories, Sparks, MD, USA) (3 g), basal media (described below) (100 mL), and deionised water (15 mL). The 20% glucose solution consisted of 20g dextrose (Difco Laboratories, Sparks, MD, USA) in 100 mL deionised water. The media (Casamino acid media, basal media and deionised water) was placed in the 500 mL flasks and autoclaved (Priorclave, Bio-Strategy Ltd) at 121ºC for 15 minutes, as was the 20% glucose solution. After autoclaving, the liquid culture flasks and glucose solution were placed in a laminar flow cabinet (ESCO® Labculture horizontal laminar flow cabinet, Bio-Strategy Ltd) until cool. Once cooled, the glucose solution was added to the flasks and the culture flasks were adjusted to between pH 4-5 by adding 0.65 mL hydrochloric acid (2N). The flasks were then ready for inoculation with the Mt isolate.

Basal Media

The basal media was prepared in a 1000 mL volumetric flask and consisted of vitamin mix stock solution (40 mL), $ZnSO_4$ stock solution (20 mL), $MnSO_4$ stock solution (20 mL), $CoCl_2$ stock solution (20 mL), KH_2PO_4 (4 g), $MgSO_4$ (0.6 g), $CaCl_2$ (0.8 g), $FeSO_4$ (0.1 g) and deionised water (900 mL).

The vitamin mix stock solution was prepared in a 1 L plastic bottle covered in aluminium foil as the vitamin solution in sensitive to light. The vitamin mix solution consisted of thiamine (25 mg), riboflavin (25 mg), ca-pantothenate (25 mg), niacin (nicotinic acid) (25 mg), pyridoamine (25 mg), thiotic acid (25 mg), folic acid (2.5 mg), biotin (2.5 mg) and vitamin B_{12} (2.5 mg) in 1 L of water.

The other three stock solutions $MnSO_4$, $ZnSO_4$ and $CoCl_2$ were prepared in 500 mL Schott bottles as follows: 0.78 g $MnSO_4$ in 500 mL water, 0.7 g $ZnSO_4$.in 500 mL water and 1.84 g $CoCl_2$ in 500 mL water.

2.2.4. Inoculating Liquid Cultures

In the laminar flow cabinet, the media flasks were inoculated by scraping the leading edge of the colony from one half of the Mt colony plate with a sterile scalpel and carefully placing the scraped fungus into the flask. After inoculation, the foil lids were put back on the flasks securely and the flasks were then placed either in a shaking incubator (Minitron® Infors incubator shaker, Bio-Strategy Ltd) at 200 rpm and 27 °C or on a shaking table in the controlled temperature (CT) room at 200 rpm and 27ºC on a 12 hr/12 hr light/dark cycle for 15 days. These cultures are hereafter referred to as the Mt culture flasks.

2.2.5. Culture Viability Tests

Culture viability tests were performed on the Mt culture flasks 13 days after inoculation to determine which culture flasks to use (i.e., those exhibiting greatest growth in biomass). There were four viability tests carried out: microsclerotia count, spore plates, dry weights and culture growth on potato dextrose agar (PDA) plates. These tests are described in more detail below.

Microsclerotia Count

Microsclerotia (ms) are hyphal aggregates that form in liquid cultures (Figure 15). On plant surfaces hyphae germinating from microsclerotia are known to establish the initial infection sites. Microsclerotia also produce spores which are vital for plant infection (Shearer, 2007; Shearer & Jackson, 2006).

In the laminar flow cabinet, 1 mL from each culture flask was placed into separate sterile 10 mL yellow-capped tubes which contained 9 mL water. The lids were replaced and the tubes inverted several times. 50 µl was pipetted onto each end of a microscope slide (100 µl in total) and covered with a coverslip. The microscope slides were placed under the light microscope (Leica DM 2500) and examined at 40× magnification. All the well-formed microsclerotia under the entire coverslip were counted and recorded.

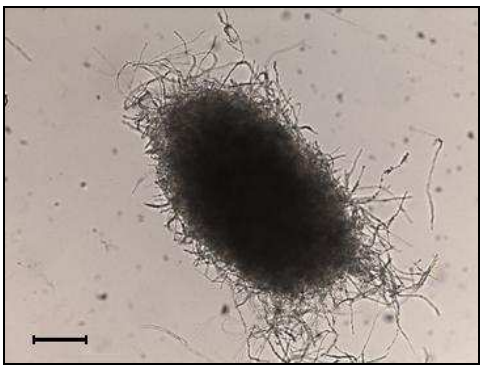


Figure 15: **Example of a well-formed microsclerotia. Scale bar = 100 µm (photograph by Author)**

Spore Plates

Spores are produced and released from the surface of microsclerotia and are responsible for secondary infection on plant surfaces (Shearer, 2007). To prepare spore plates in the laminar flow cabinet, 1 mL of the 10 fold dilution in the yellow capped tubes prepared for the microsclerotia counts was pipetted on to two water agar (WA) plates. The plates were lightly shaken horizontally to spread the diluted culture over the plate. The plates were then placed in a clear plastic box in the controlled-temperature (CT) room (12 hr/12hr light/dark cycle, 27 ºC). After seven days the spores on the plates were counted using a haemocytometer. Water (1 mL) was placed onto each spore plate and gently spread around the plate using a sterile bacterial loop to dislodge any spores. Water from the plate was pipetted (ca. 100 µl) and placed in the middle of the haemocytometer and covered with the coverslip. The haemocytometer was placed under the light microscope and examined at 100× magnification. The spores present were counted and recorded from 4 x 16 squares from each chamber (Figure 16).

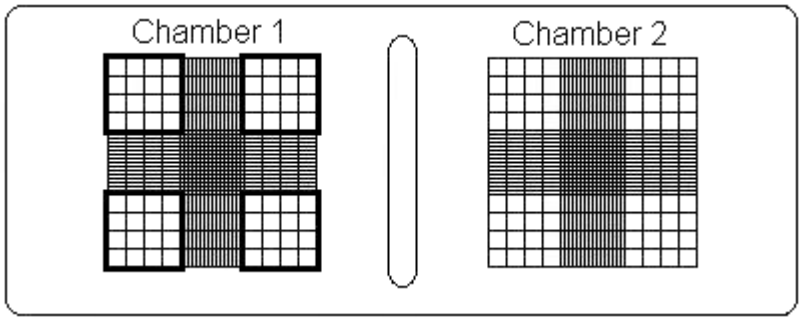


Figure 16: **Illustration of a haemocytometer. Spores counted in the 4 x 16 squares in the corners (bolded outline) in both chambers. Block of 16 squares = 1 mm^2**

Dry Weights

Changes in the dry weight of the culture are important as they indicate the liquid culture viability through fungal growth, i.e., biomass accumulation of hyphae, mycelium and microsclerotia (Jackson, 1997; Shearer, 2002). From the dry weights (actual Mt biomass in the liquid culture) the volume of culture required for each mycoherbicide treatment dose was calculated.

From each culture flask, 1 mL of the fungal biomass (hyphae, mycelium and microsclerotia) was filtered through pre-weighed filter paper in a Buchner funnel attached to a vacuum flask. After filtering, the filter paper was placed into an aluminium dish and dried to constant weight in a 60 ºC drying oven (Contherm Digital series oven, Bio-Strategy Ltd). The dry weight of the filter paper and culture was recorded and the biomass of Mt in the culture was determined.

Culture Growth on PDA Plates

Plating back liquid culture onto agar plates tests both the viability of the Mt biomass through re-isolation of Mt on agar plates and the liquid culture purity, i.e., for contamination by other fungi or bacteria.

From each culture flask 500 µl of culture was placed in the centre of two PDA plates. The plates were placed in a clear plastic box in the CT room (12 hr/12hr light/dark cycle, 27 ºC) and examined periodically to ensure the cultures were growing on the plates and that the plates were clean, i.e., no sign of contamination by other microbes (Figure 17).

Figure 17: **Example of liquid culture plated back onto PDA. Plate diameter = 90 mm (photograph by Author)**

2.2.6. Preparation of Mycoherbicide Inoculum

Once the liquid culture biomass was determined (24-48 hr) in the culture flasks, the flasks were filtered (Whatman hardened ashless filters, grade 540) in a Buchner funnel attached to a vacuum flask. After filtering, the Mt fungal mat on the filter paper was placed in a Schott bottle (Labserv®) and re-suspended in the same volume of water as the filtrate (Figure 18).

Figure 18: **500 mL Schott bottle containing Mt fungal mat in re-suspended water (photograph by Author)**

2.2.7. Dose Determination

The chosen treatment doses for the experiments were based on the top dose (30 mg Mt/L) known to be effective on alien plants used in a mesocosm trial done by NIWA. The four chosen dose concentrations were 60, 30, 15 and 7.5 mg Mt/L. Not all dose concentrations were used in each experiment and each experiment had a fresh batch of liquid cultures. In order to determine the volume of liquid culture required for the inoculation, the liquid culture biomass, the volume of the experimental container and the concentration of Mt required for that volume of container has to be known. For example, if the experimental container is a 2L jar and the dose concentration is 30 mg Mt/L then 60 mg Mt is required to inoculate the 2 L jar. If the liquid culture biomass is 20 mg Mt/mL then 3 mL of the liquid culture is required to inoculate the 2 L jar.

2.3. Experiment 1: Lake Seed Bank Germination

2.3.1. Materials and Methods

Mycoherbicide Preparation

Preparation of the mycoherbicide inoculum is detailed in Section 2.2.1-2.2.3. The volume of mycoherbicide inoculum required for each of the four mycoherbicide doses was calculated based on the amount of Mt required (mg) per 2 L in glass jars and the concentration of Mt (mg/mL) in the liquid culture (Table 6).

Table 6: Mycoherbicide inoculum calculations

Dose required (mg/L)	Mt weight required per 2L (mg)	Mt culture concentration (mg/mL)	Culture volume required for inoculation (mL)
60	120	14.2	8.45
30	60	14.2	4.23
15	30	14.2	2.11
7.5	15	14.2	1.06

Herbicide Preparation

Herbicide concentrations are based on the percentage active ingredient (AI) of each herbicide as listed on the herbicide label (Table 7). Four dose rates for each herbicide were chosen, an upper concentration representing the maximum recommended label rate, and three doses across an increasing dilution series (Table 8). Latex gloves, breathing apparatus and safety eyewear were used when handling concentrated herbicides.

Table 7: **Active ingredient components and percentages for each herbicide according to the herbicide label (A) and the active ingredients concentration calculated from the AI percentage (B)**

A

Herbicide	Active Ingredient (%)	Active Ingredients
Reglone (Diquat)	20%	Diquat cation or diquat dibromide
Aquathol K (Endothall)	40.30%	Dipotassium salt of endothall (endothall acid a.e.)
Sonar AS (Fluridone)	41.70%	1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1H)-pyridinone
Chelated Copper (K-Tea) (experiment 4 only)	8%	Copper as elemental (derived from copper-triethanolamine complex and topper hydroxide)

B

Diquat	Endothall	Fluridone	Chelated Copper (experiment 4 only)
20 % in 100 % 200 g in 1000 ml 200 g/L = 200000 ppm	40.3 % in 100 % 403 g in 1000 ml 403 g/L = 403000 ppm	41.7 % in 100 % 417 g in 1000 ml 417 g/L = 417000 ppm	8 % in 100 % 80 g in 1000 ml 80 g/L = 80000 ppm

For each herbicide a 50 ppm working solution was first made in a 5 L plastic container. This was achieved by pipetting (epResearch®, Eppendorf) 0.5 mL of each herbicide into the appropriate calculated volume of water (Table 16, Appendix 1). The volume of water required was calculated using the formula below.

$$\text{Diluted Solution Volume} = \frac{\text{Concentrated Solution Concentration} \times \text{Concentrated Solution Volume}}{\text{Diluted Solution Concentration}}$$

E.g.,Example calculation for diquat

$$\text{Diluted Solution Volume} = \frac{200\,000 \text{ ppm} \times 0.5 \text{ mL}}{50 \text{ ppm}}$$

$$\text{Diluted Solution Volume} = 2\,000 \text{ mL}$$

This experiment was carried out in 2 L jars, with each dose having 5 replicates. The four different doses required for each herbicide (Table 8) were prepared from the 50 ppm working solution in 10 L plastic buckets (5 x 2 L jars requires 10 L of herbicide dose solution). Each herbicide had a dedicated 10 L bucket with doses sequentially prepared from most dilute to most concentrate. The volume of concentrated working solution required to make 10 L of each dilution was calculated using the formula below.

$$\text{Concentrated Solution Volume} = \frac{\text{Diluted Solution Concentration} \times \text{Diluted Solution Volume}}{\text{Concentrated Solution Concentration}}$$

E.g. Example calculation for 2 ppm diquat

$$\text{Concentrated Solution Volume} = \frac{2 \text{ ppm} \times 10\,000 \text{ mL}}{50 \text{ ppm}}$$

$$\text{Concentrated Solution Volume} = 400 \text{ mL}$$

Table 8: **Treatment doses for each herbicide starting with the maximum label rate (1) and decreasing in concentration across a dilution series (2-4).**

Herbicide	Doses			
	1	2	3	4
Diquat	2 ppm	1.5 ppm	1 ppm	0.5 ppm
Endothall	5 ppm	3.75 ppm	2.5 ppm	1.25 ppm
Fluridone	0.15 ppm	0.1 ppm	0.05ppm	0.005 ppm

Experimental Set-up and Inoculation

120 mL mixed seed bank material (mixing ratio A, Table 5, Chapter 2.1.4) was placed into each 130 mL plastic sample container (LabServ®). In total there were 90 plastic containers filled with sediment: 85 for the experiment and 5 for determining background germination rates to guide the timing for harvesting of the experiment. Each plastic container with seed bank material was placed into a 2 L glass jar (22.8 cm in height) (Arthur Holmes Ltd) filled with the appropriate herbicide solution or water. There were four treatment concentrations per herbicide (diquat, endothall and fluridone, Table 8) and mycoherbicide (Table 6), which were replicated five times. There were also five untreated control jars. The jars were set up on a table under controlled light (14 hr/10 hr light/dark cycles) and temperature conditions in a Constant Temperature (CT) room (20ºC). All treatments and controls were randomly assigned to the 2 L jars and to their positions on the table. Once the jars were in place, a temperature / light logger (Onset HOBO® pendant temperature / light logger) was placed into one of the jars and an aeration frame (made by Jim Patmore, NIWA) and aeration tubes (5 mm diameter) were submerged in the solution of each jar (Figure 19).

The temperature remained constant in the jars throughout the experiment at 21.8 ºC. The light intensity decreased slightly towards the end of the experiment. However, the average PAR (photosynthetically active radiation) intensity for the light period was 30.4 μmol/m^2/s.

Aeration (air compressor,(Brooklands V.I.P Pet Products, New Zealand)) was started after 24 hr to allow the mycoherbicide to settle. The evaporative losses in the jars were topped up weekly with the appropriate herbicide solution. Only a very small volume (ca 50-100 mL) was used for topping up each time.

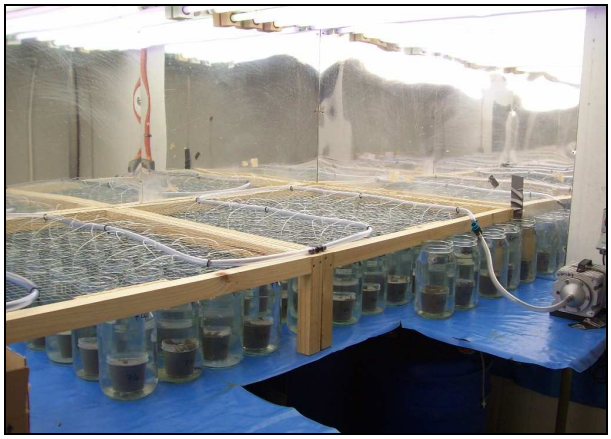


Figure 19: **Experimental layout of inoculated jars with lake sediment in the CT room with the aeration frame in place (photograph by Author)**

Harvesting

The five jars for background germination rates were harvested (i.e. emerged germlings above the sediment were carefully removed, counted and identified) at different time intervals to determine when there were sufficient germlings present (20+) for all the jars to be harvested (ca. 35 days).

Harvesting of the experiment jars 36 days after treatment involved a systematic strategy, so as to remove potential timing bias. This was done by harvesting successive replicates (replicate 1 followed by replicate 2, replicate 3, etc.) from each herbicide (diquat, endothall and fluridone) and mycoherbicide and the control. Each sediment pot was placed under a cold light source (Leica CLS 150X) and emerged germlings (visible germlings above the sediment) were carefully removed, together with the attached oospore where possible, and placed into glass petri dishes. The germlings were then identified to species level under a stereo microscope (Leica MZ 9.5) (up to 60× magnification) using vegetative and oospore characteristics, counted and recorded.

Statistical Analyses

Comparison between dose rates and the control treatment:

An ANOVA (GenStat 12th Edition) was used to identify any significant differences ($p < 0.05$) and linear trends within the herbicide dose rate series. Two post-hoc tests (t–distribution test and Dunnett's test) were used to distinguish any significant differences between the control and each of the treatment doses for each herbicide.

2.4. Experiment 2: Sieved Oospore Germination

2.4.1. Materials and Methods

Mycoherbicide Preparation

Preparation of the mycoherbicide inoculum is detailed in Section 2.2.1-2.2.3. The volume of mycoherbicide inoculum required for each of the 4 mycoherbicide doses was calculated based on the amount of Mt (mg) required per 0.5 L in plastic containers and the mg/mL concentration of cultured Mt (Table 9).

Table 9: Mycoherbicide inoculum calculations

Dose required (mg/L)	Mt weight required in 0.5L (mg)	Mt culture concentration (mg/mL)	Culture volume required for inoculation (mL)
60	30	19.08	1.57
7.5	3.75	19.08	0.197

Herbicide Preparation

The herbicide preparation calculations are the same as in Experiment 1 and the dilution table for each of the herbicide treatment doses can be found in Table 17, Appendix 1. For this experiment there were only two concentrations used for each herbicide: diquat (0.1 ppm and 2 ppm), endothall (0.1 ppm and 5 ppm) and fluridone (0.005 ppm and 0.15 ppm). For each herbicide a 50 ppm solution was made first in 10 L containers. The 2 different herbicide doses required for each herbicide were made in 25 L jerry cans from the 50 ppm solution to allow for half of the herbicide and water to be exchanged daily in each of the experimental containers. Each herbicide and dose had its own jerry can (6 x 25 L jerry cans).

Experimental Set-up and Inoculation

120 mL Mixed seed bank material (mixing ratio B, Table 5, Chapter 2.1.4) was measured and carefully sieved through 250 µm and 500 µm sieves. The oospores and debris collected in the sieves were placed into a large glass petri dish and any floating debris was decanted off. The contents of the petri dish were then emptied into a 500 mL container and the appropriate herbicide treatment was added.

In total there were 50 containers (500 mL plastic) with sieved sediment, 45 for experiment and 5 for determining background germination rates prior to harvesting the main experiment. There were 2 treatment concentrations per herbicide; diquat (0.1 ppm and 2 ppm), endothall (0.1 ppm and 5 ppm) and fluridone (0.005 ppm and 0.15 ppm) and mycoherbicide (7.5 mg/L and 60 mg/L, Table 9) and one control. Each treatment and control was replicated 5 times. All treatments and controls were randomly assigned to the plastic containers. The containers were set-up under controlled light (14 hrs/10 hrs light/dark cycles) and temperature conditions (20 °C) in the CT room. A temperature and light logger was placed in an extra 500 mL plastic container filled with water and placed adjacent to the experimental containers (Figure 20).

The temperature remained constant in the jars at 21.20 ºC throughout the experiment. Initial low light at the beginning of the study was due to positioning of the logger. Initially the probe remained in a 2 L jar from experiment 1 for 8 days then was subsequently placed in the elevated 500 mL water container (same height as experimental containers) where it stayed throughout the experiment. The

average light period when the probe was in the correct position was 122.74 μmol/m^2/s.

Figure 20: **Experimental layout of the treated sieved lake sediment, the 5 extra containers and the container with the temperature and light probe (photograph by Author)**

Herbicide/Water Exchange

A peristaltic pump (Watson-Marlow 504S) had initially been planned to keep the concentration constant in the 500 mL containers. However, the tubing stream divider system (Pulse Instrument Ltd) set-up did not allow the pumping to work evenly and the containers would have received different volumes of the herbicides. Given the experimental timeframe, constant concentrations were instead maintained manually by daily herbicide (diquat, endothall and fluridone) and water exchange (mycoherbicide and control) in treatment containers. Each container had the appropriate half herbicide and/or water content replaced daily to ensure a sustained treatment concentration of products throughout the study. This was carried out by carefully siphoning out half the contents (250 mL) from each

container into a waste bucket. The experimental containers then had the appropriate herbicide and water siphoned slowly (to ensure minimal disturbance within the container) back into the containers to replace the removed solution (Figure 21). The herbicides used for herbicide replacement came from the 'stock solutions' made in the jerry cans.

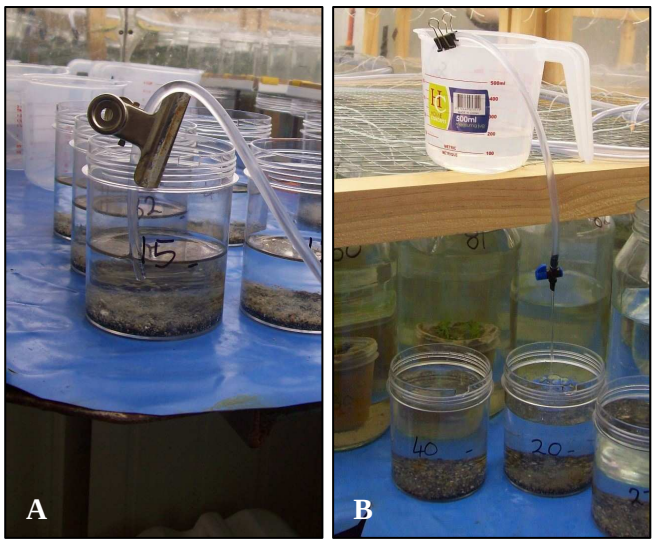


Figure 21: **Herbicide and water removal (A) and replacement (B) (photographs by Author)**

Harvesting

The 5 extra containers were harvested (i.e. germinated oospores were carefully removed from debris using tweezers, counted and identified) at different time intervals to determine when sufficient germlings were present (100+) for all the experimental containers to be harvested (ca. 33 days).

Harvesting of the oospores from each 500 mL container 34 days after treatment was systematic, so as to remove any potential timing bias. This was done by harvesting successive replicates (replicate 1 followed by replicate 2, replicate 3

etc) from each treatment and the control containers. Germinated oospores were carefully removed from the containers by gently sifting through the debris using tweezers under the stereo microscope and placed into glass petri dishes. The germlings were then identified to species level under a stereo microscope (up to 60× magnification) using vegetative and oospore characteristics, counted and recorded.

Statistical Analyses

Comparison between dose rates and the control treatment:

ANOVA (GenStat 12th Edition) was used to identify any significant differences ($p < 0.05$) within each treatment dose for each herbicide. Two post-hoc tests (t – distribution test and Dunnett's test) were used to distinguish any significant differences between the control and each of the treatment doses for each herbicide.

2.5. Experiment 3: Outdoor Germling Response

2.5.1. Materials and Methods

Mycoherbicide Preparation

Preparation of the mycoherbicide inoculum is detailed in Section 2.2.1-2.2.3. The volume of mycoherbicide inoculum required for each of the 4 mycoherbicide doses was calculated based on the amount of Mt (mg) per 120 L in tanks required and the mg/mL concentration of cultured Mt (Table 10).

Table 10: Mycoherbicide inoculum calculations

Dose required (mg/L)	Mt weight required per 120 L (mg)	Mt culture concentration (mg/mL)	Culture volume required for inoculation (mL)
60	7200	12.8	564.71

Herbicide Preparation

Herbicide preparation calculations are the same as those in Experiment 1. However, for this experiment there was only one concentration (maximum label rate) for each herbicide: diquat (2 ppm), endothall (5 ppm) and fluridone (0.15 ppm). The calculated volume of herbicide (Table 11) was added directly to 120 L tanks.

Table 11: Dilution table for herbicide treatment doses based on concentration of active ingredient (AI)

Herbicide	Diluted Solution Concentration. ppm AI	Diluted Solution Volume. mL	Concentrated Solution Concentration. ppm AI	Concentrated Solution Volume. mL
Diquat	2	120 000	200 000	1.200
Endothall	5	120 000	403 000	1.489
Fluridone	0.15	120 000	417 000	0.043

Experimental Set-up and Inoculation

120 mL mixed 'seed bank' material (mixing ratio A, Table 5, Chapter 2.1.4) was placed into each 130 mL plastic sample container. In total there were 300 sample containers filled with sediment. All the sample containers with seed bank material were placed in the outdoor water trough (NIWA Ruakura) (Figure 23) to germinate and grow into well established germlings (12.5 weeks preculture). A water temperature and light logger was placed in the trough and the trough was covered with 90 % shade cloth (R.J Reid Ltd, Auckland, New Zealand).

The temperature remained constant at 21.2 ºC in the preculture trough during the initial germination and growth of the charophyte germlings. The light intensity varied slightly reflecting changing ambient radiation. The daylight hours decreased with the changing of the seasons from summer to autumn (Figure 22).

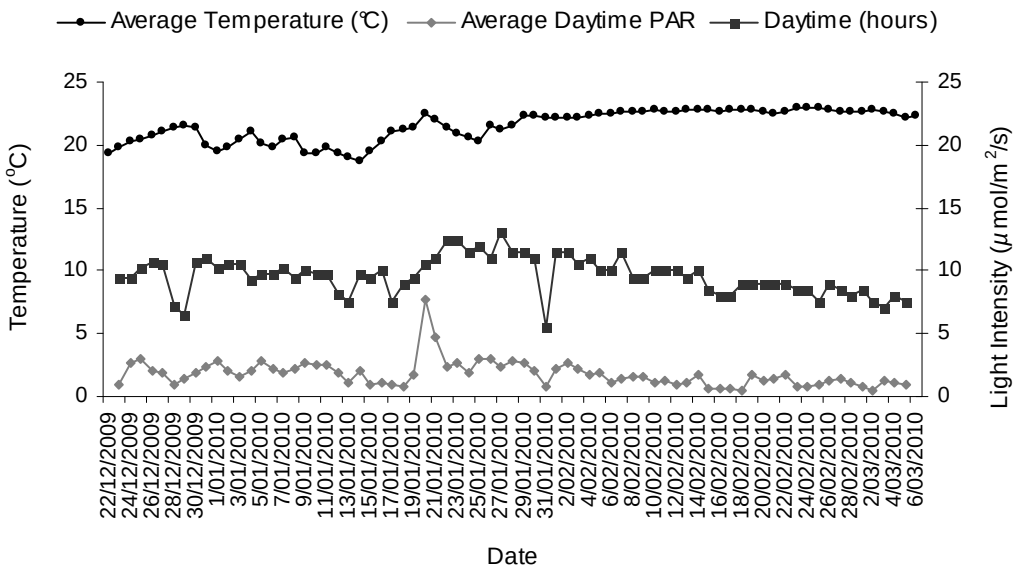


Figure 22: **Temperature, daytime PAR (μmol/m^2/s) and daytime hour profiles recorded in the preculture trough covered with 90% shade cloth for 75 days**

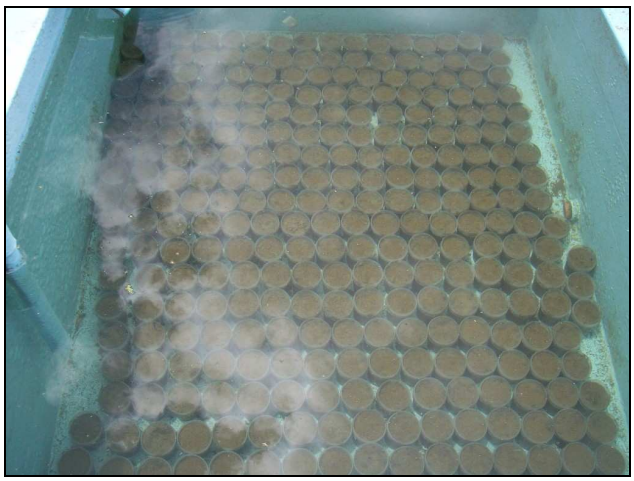


Figure 23: **Sediment pots within the concrete trough for charophyte germination and germling establishment prior to treatment (photograph by Author)**

At the start of the experiment, 10 random germling pots were removed to establish pre-treatment biomass of the charophyte germlings. The remaining germling pots were relocated into 120 L tanks with 10 germling pots placed in each tank. The germling pots were placed in the tanks in a systematic way whereby one pot was placed into each of the 25 tanks then the second pot was placed into each tank and

so on until 10 pots were in each tank (Figure 24). This prevented any potential tank bias by separating adjacent pots from the preculture trough. The tanks were located in an open greenhouse in NIWA's compound at Ruakura.

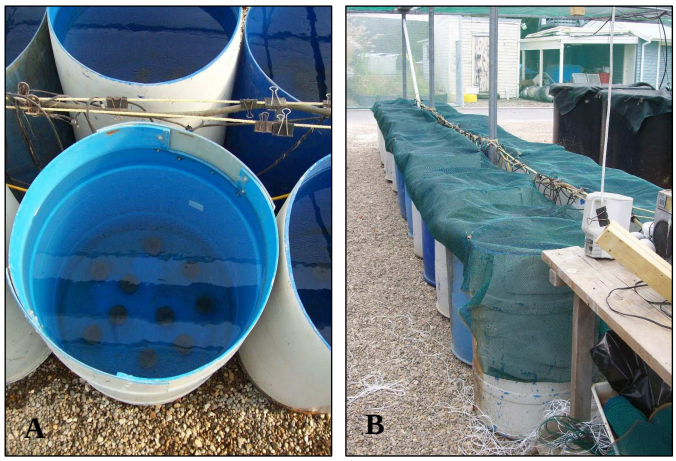


Figure 24: **Placement of 10 germling pots in one 120 L tank (A). Experimental layout of the 120 L tanks covered with 50 % shade cloth in NIWA greenhouse (B). (photographs by Author)**

Treatments and controls were randomly assigned to each of the 25 120 L tanks, with 5 replicates for each herbicide (diquat, 2 ppm; endothall, 5 ppm; fluridone, 0.15 ppm), the mycoherbicide (60 mg/L) and the controls. After treatment, temperature and light loggers were placed into two different tanks. The water temperature declined over the 10 week period from around 20 ºC to 14 ºC (Figure 25). There was little to no variation in the water temperature between the different tank positions. Light intensity under the combined shade of the greenhouse and shade cloth averaged between 1.5 and 2 PAR and varied somewhat according to the position of the tanks (Figure 25). The daylight hours steadily decreased with the changing of the seasons from autumn to winter.

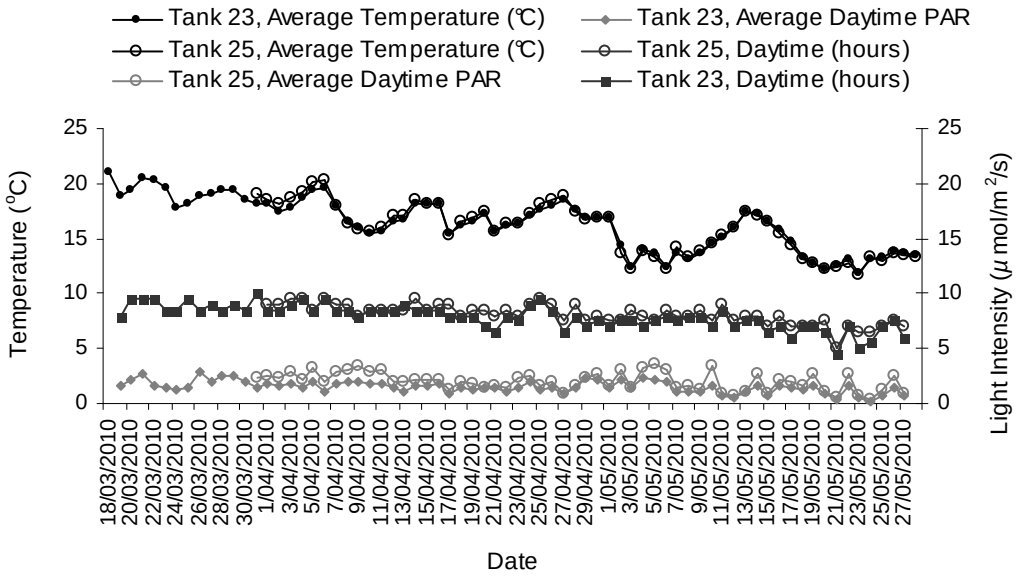


Figure 25: **Temperature, daytime PAR (μmol/m^2/s) and daytime hour profiles recorded in the 120 L tanks under the greenhouse in the middle (Tank 23) and end (Tank 25) tanks over the 10 week experimental period**

The tanks were covered with 90 % shade cloth (Figure 24 B) and aerated by compressor via 5 mm tubing and airstones. The experiment ran for 10 weeks with two harvests of 5 pots from each tank, one at 5 weeks and the other at 10 weeks. Weekly visual observations (height, plant health, general tank conditions, i.e. water clarity and algal growth) were performed on each tank over the 10 week period.

Harvesting

At 5 weeks and 10 weeks, 5 germling pots were randomly selected and removed from each tank. The pots were placed under the Lecia light source and visible germlings were carefully removed using tweezers. The germlings from each pot were rinsed and placed in the centre of a pre-weighed tinfoil sheet (Caterers Foil, James Gilmour & Co Ltd, Mt Roskill) which was then folded securely. The foil sheets were placed into an 80 °C drying oven (Contherm Thermotec 2000 series

oven, Bio-Strategy Ltd) and dried to constant weight (more than 24 hrs). The foil sheets were weighed (± 0.0001 g), initial foil weight deducted, and the charophyte biomass determined and recorded.

Statistical Analysis

Comparison between herbicide treatments:

An ANOVA (GenStat 12th Edition) was used to identify any significant differences ($p < 0.05$) between the different treatments (control, diquat, endothall, fluridone and mycoherbicide) and two post-hoc tests (Tukey test and Dunnett's test) were used to distinguish any significant differences between the control and each of the treatments.

2.6. Experiment 4: Germling Response to Herbicides and Algaecide

2.6.1. Materials and Methods

Mycoherbicide Preparation

Preparation of the mycoherbicide inoculum is detailed in Section 2.2.2-2.2.4. The volume of mycoherbicide inoculum required for each of the 4 mycoherbicide doses was calculated based on the amount of Mt (mg) in 2 L glass jars required and the mg/mL concentration of cultured Mt (Table 12).

Table 12: Mycoherbicide inoculum calculations

Dose required (mg/L)	Mt weight required per 2 L (mg)	Mt culture concentration (mg/mL)	Culture volume of required for inoculation (mL)
60	120	31.9	3.760
26.25	52.5	31.9	1.645
7.5	15	31.9	0.470

Herbicide Preparation

The herbicide preparation calculations were the same as those in Experiment 1 and the dilution table for each of the herbicide treatment doses can be found in Table 18, Appendix 1. For this experiment a chelated copper algaecide (K-Tea) was also used.

For each herbicide a 50 ppm solution was made in 5 L containers first. The 3 treatment doses required for each herbicide (Table 13) were made from the 50 ppm solution in 10 L plastic buckets (5 x 2 L jars require 10 L of herbicide dose

solution). Each herbicide had a dedicated 10 L bucket with doses sequentially prepared from most dilute to most concentrate.

Table 13: Treatment doses for each herbicide and algaecide starts with the max label rate (1) and decreasing in concentration across a dilution series (2-3)

Herbicide	Doses		
	1	2	3
Diquat	2 ppm	1.05 ppm	0.1 ppm
Endothall	5 ppm	2.55 ppm	0.1 ppm
Fluridone	0.15 ppm	0.0775 ppm	0.005 ppm
K-Tea	1 ppm	0.75 ppm	0.5 ppm

Experimental Set-up and Inoculation

120 mL mixed seed bank material (mixing ratio B, Table 5, Chapter 2.1.4) was measured and placed into each of 130 plastic sample containers (Labserv®). The sample containers were placed into 2 water-filled containers (110 L storage organiser containers). Ten days later 60 plastic sample containers were filled with 120 mL mixed seed bank material each and 3 x 15 cm *L. major* apical shoots were planted into the sediment of each container (Figure 26). The 10 day time delay was to reduce the amount of *L. major* shoot growth while the sediment pots germinate. If the shoots grow too much in height they may not be able to fit in the experimental jars. The *L. major* pots were placed into a water- filled container (60 L storage organiser container) until the container was full and the remaining unplaced *L. major* pots were placed into open spaces in the two germling pot containers (Figure 26). The 3 water containers were placed in the CT room (14 hr/10 hr light/dark cycle, 20 ºC), aerated and a water temperature and light logger was placed in each. All 190 containers remained in the 3 water filled containers

until the oospores had germinated (42 days). The temperature remained constant at 19.56 ºC in the containers. The average light period was 5.02 μmol/m^2/s.

Figure 26: **Sediment pot preparation (A) and placement of pots in water containers in the CT room (B) (photographs by Author)**

80 germling pots were placed into 2 L jars after germling height above the sediment surface was recorded and 30 *L. major* pots were placed into 2 L jars after *L. major* shoot heights above ground were recorded. 20 remaining *L. major* pots were used for pre–treatment biomass measurements (see Harvesting below), however, pre–treatment biomass of charophytes was not measured as it was negligible.

For the charophyte germling jars there were 3 treatment concentrations per herbicide: diquat, endothall fluridone (Table 13), mycoherbicide (Table 12) and algaecide (chelated copper) (Table 13) and untreated control. For the *L. major* jars there was only one treatment dose (max label rate) per herbicide (diquat, endothall, fluridone mycoherbicide) and algaecide (chelated copper) (Table 12-13). Each treatment and control was replicated 5 times. All treatments and

controls were randomly assigned to the 2 L jars. The jars were set-up under controlled light (14 hr/10 hr light/dark cycles with the average light period in the experimental jars of 36.60 μmol/m^2/s PAR) and temperature (averaged 20.34 °C) conditions and once the jars were in place, a temperature and light logger was placed into two separate jars and the aeration frame (see Chapter 2) was put in place. Aeration was started after 24 hrs to allow the mycoherbicide to settle (Figure 27).

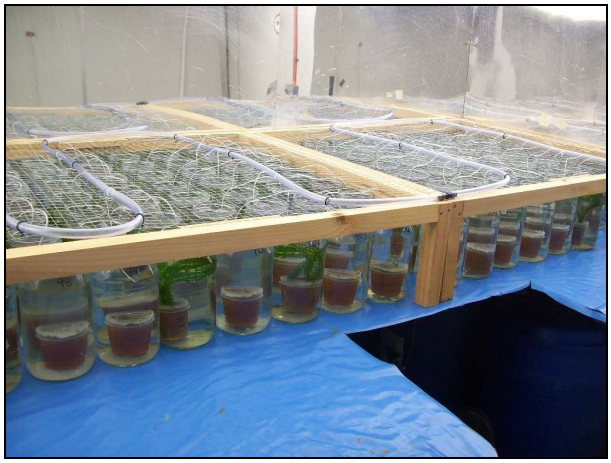


Figure 27: **Experimental set-up of the 2 L glass jars with sediment pots and *L. major* pots in the CT room after inoculation (photograph by Author)**

Harvesting

Plants were harvested after 14 days. Harvesting of the germling biomass and *L. major* biomass in each 2 L jar involved a systematic strategy, so as to remove any potential timing bias, by harvesting successive replicates (replicate 1 followed by replicate 2, replicate 3 etc) from each herbicide (diquat, endothall, fluridone and mycoherbicide) and algaecide (K-Tea) and the control. Harvesting of the experiment involved removing the germling pot or *L. major* pot from each jar and measuring the height of the charophyte germling and *L. major* shoots above ground. The germling biomass was placed onto pre-weighed (± 0.0001 g) foil

(Caterers Foil, James Gilmour & Co Ltd, Mt Roskill) sheets while *L. major* shoots were placed into separate brown paper bags (No. 0 block bottom (heavy duty), E C Attwood Ltd, Manakau). The foil sheets and paper bags were placed into an 80 °C drying oven (Contherm Thermotec 2000 series oven, Bio-Strategy Ltd) and dried to constant weight (more than 24 hrs). The foil sheets were weighed (± 0.0001 g), initial foil weight deducted, and the charophyte biomass determined and recorded. The *L. major* shoots were weighed (± 0.0001 g) (without bag) and recorded.

Statistical Analysis

Comparison between dose rates and the control treatments:

ANOVA (GenStat 12th Edition) was used to identify any significant differences ($p < 0.05$) within each treatment dose for each herbicide, and two post-hoc tests (t – distribution test and Tukey test) were used to distinguish any significant differences between the control and each of the treatment doses for each herbicide.

Chapter 3

3. Experiment 1: Lake Seed Bank Germination

3.1. Introduction

The objective of this chapter is to examine whether the three herbicide and mycoherbicide products negatively impacted oospore germination from lake sediment and whether sensitivity differed between charophyte species. The experimental hypothesis is that charophyte germination will not be affected by herbicide or mycoherbicide treatments at concentrations representing potential application rates.

This experiment involved placing aliquots of mixed seed bank material into glass jars and treating with three herbicides (diquat, endothall and fluridone), a mycoherbicide and an untreated control. Each herbicide and mycoherbicide treatment had four dose concentrations which were applied as a pre–emergent application.

3.2. Results

Mycoherbicide Culture

The liquid culture used for inoculation had a microsclerotia count of 2.7 x 10^{-3}/mL, a biomass of 14.2 mg/mL and a zero spore count. The liquid culture placed onto PDA plates grew with no sign of contamination (Table 19-22, Appendix 2).

Experimental Harvests

Observations

Two weeks after treatment, the colour in charophyte jars treated with the mycoherbicide was yellow-green, indicative of algal growth compared to the rest of the treated charophyte jars (Figure 28). The top dose of the mycoherbicide treated jars generally had the darkest yellow-green colour development.

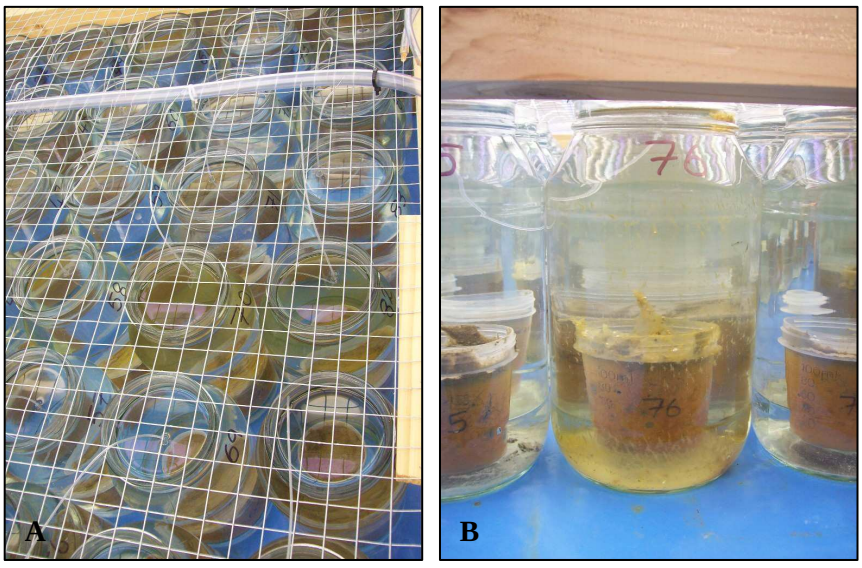


Figure 28: **Example observations of water colour two weeks after treatment. (A) Colour difference of Jars 58, 70 and 82 which were treated with 7.5, 30 and 15 mg/L of mycoherbicide respectively. (B) Jar 76, treated with 60 mg/L of mycoherbicide, with yellow algae growth (photographs by Author)**

Germination and Species Count

The germination for the mycoherbicide treatment had a lower overall germination count than all the other treatments (except the Control) and endothall-treated jars had the highest germination count (Figure 29, Table 14). There were two treatments; diquat and endothall, which showed a linear trend of decreasing germination count with increasing treatment dose. However, there was a general tendency for the highest dose in most treatments (excluding the mycoherbicide) to have a lower germination count. According to post hoc tests (t-distribution test), there were two treatment doses; endothall 1.25 ppm and fluridone 0.1 ppm, which had a significantly higher germination count than the control (Figure 29).

For the species count there were no linear trends across the treatment dose series (Table 14) and there were no significant differences between the control and any of the treatment doses according to the t - tests (Figure 29).

Table 14: Statistical analysis of linear trends (ANOVA $p < 0.05$) across the dose series

Treatment	Germination count	Species count
Diquat	**0.038**	0.555
Endothall	**0.045**	0.709
Fluridone	0.293	0.524
Mycoherbicide	0.302	0.312

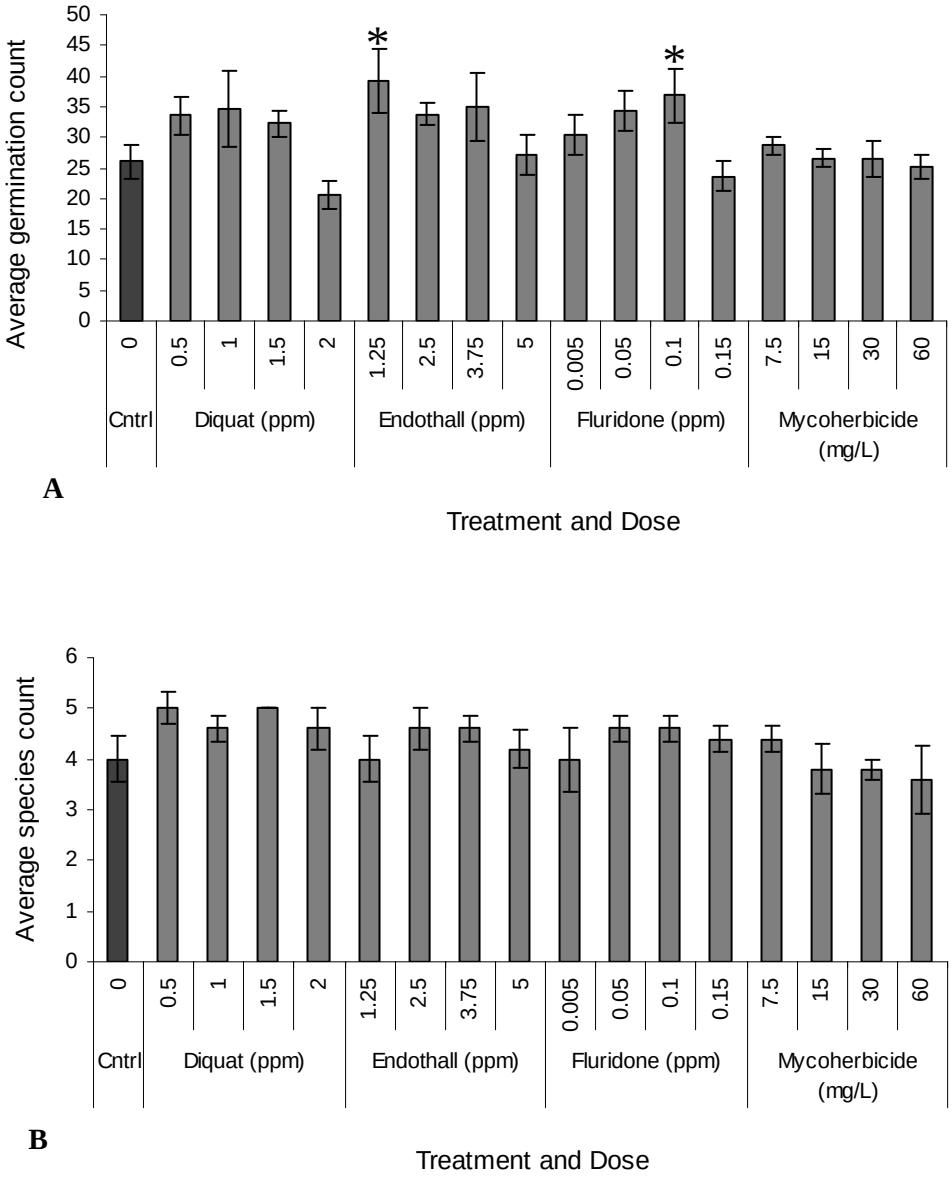


Figure 29: **Germination (A) and species (B) count within all treatments. * = t-distribution test p < 0.05, * = Dunnetts test p < 0.05. Cntrl is control. Treatment dose means +/- 1 SE**

Species Sensitivity

N. leonhardii

The germination count in all treatments had either a similar or lower germination count (mycoherbicide and endothall treatment jars) when compared to the control jars (Figure 30). This is supported by the t-distribution test when each of the treatment doses were compared with the control, showing there were 5 herbicide doses; endothall 3.75 and 5 ppm, and mycoherbicide 15, 30 and 60 mg/L, which had significantly lower germination counts than the control (Figure 30). There was a lot of variation between the replicates (i.e. the high standard error of the means). Endothall was the only herbicide to show a significant relationship with dose, but this was a decreasing linear trend with increasing dose amount (Lin $p < 0.05$).

N.* aff. *cristata

The germination count for each herbicide treatment was similar or higher than the control (Figure 30). There were no linear trends within each treatment dose (Table 15). Fluridone at 0.05 ppm had a significantly higher (t- distribution test) germination count than the control case (Figure 30).

N. hyalina

The germination count for *N. hyalina* was the highest in the endothall treatments while the mycoherbicide treatments had the lowest germination count (except the control) (Figure 31). However, there were no linear trends within each treatment ($p > 0.05$) (Figure 31, Table 15). In comparison with the control (t- distribution test) there were four treatment doses; endothall 1.25, 2.5, 3.75 ppm and fluridone

0.1 ppm, which had a significantly higher germination count than the control (Figure 31).

N. pseudoflabellata

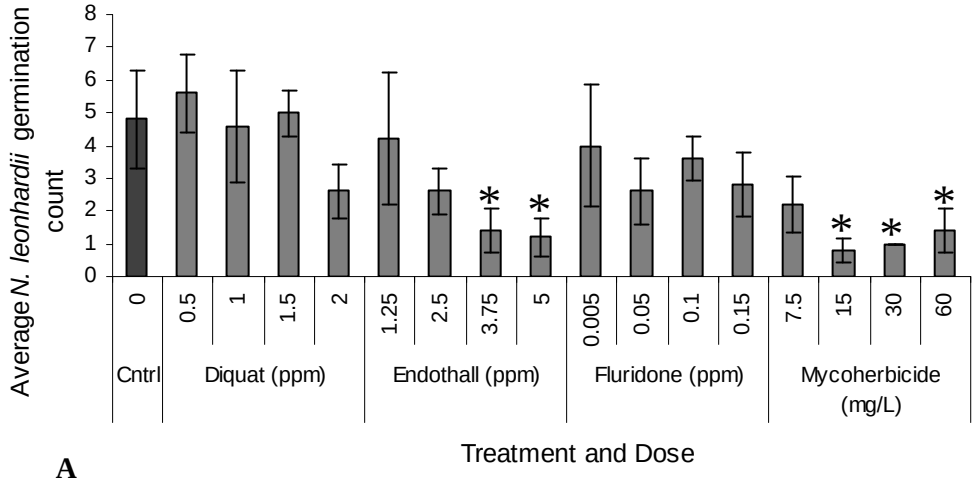
The *N. pseudoflabellata* germination count was higher in the diquat, endothall and fluridone treatments than the mycoherbicide treatments and the control jars (Figure 31). There were no linear trends within each treatment (Table 15). However, diquat at 1 ppm, endothall at 2.5, 3.75, 5 ppm and fluridone at 0.005, 0.1 ppm, were significantly different (increased germination count) to the control using the t- distribution and/or Dunnett's test (Figure 31).

C. globularis

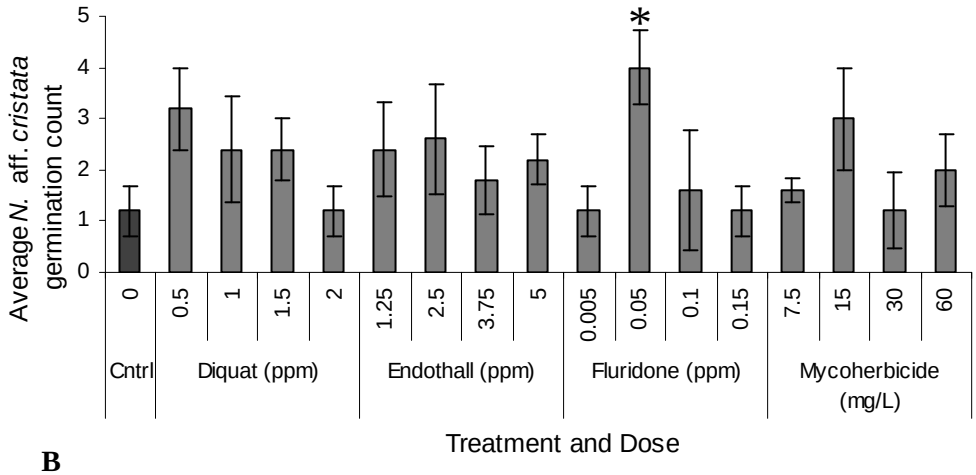
There was generally a lower *C. globularis* germination count in the diquat and fluridone treatments compared with the endothall treatment. There were two treatments, endothall and fluridone, which had a decreasing linear trend with increasing dose amount (Table 15). The top doses in each treatment generally had a lower germination count. According to the t-distribution test, there were only two treatment doses, diquat 2 ppm and endothall 1.25 ppm, which had significantly lower and higher germination counts respectively, than the control (Figure 31).

Table 15: Statistical analysis of linear trends (ANOVA p < 0.05) across the dose series

Treatment	*Nitella leonhardii*	*Nitella* aff. *cristata*	*Nitella hyalina*	*Nitella pseudoflabellata*	*Chara globularis*
Diquat	0.173	0.095	0.176	0.921	0.054
Endothall	**0.048**	0.701	0.150	0.250	**0.046**
Fluridone	0.656	0.440	0.991	0.277	**0.017**
Mycoherbicide	0.632	0.827	0.194	0.838	0.619



A



B

Figure 30: Germination count within all treatments. (A) *N. leonhardii,* and (B) *N.* aff. *cristata*. * = t-distribution test p < 0.05, * = Dunnetts test p < 0.05. Cntrl is control. Treatment dose means +/- 1 SE

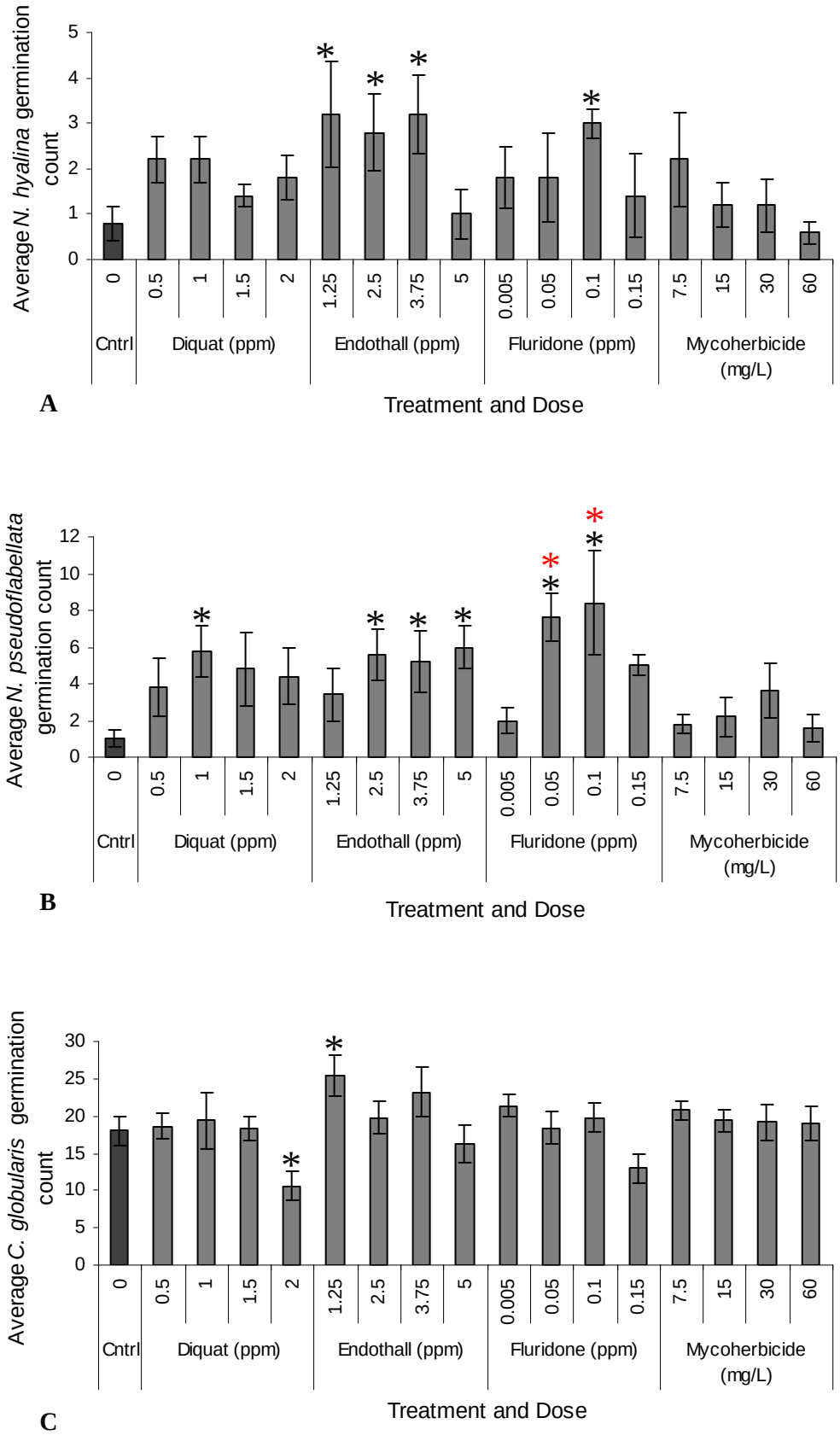


Figure 31: **Germination count within all treatments. (A) *N. hyalina*, (B) *N. pseudoflabellata* and (C) *C. globularis*. * = t-distribution test p < 0.05, * = Dunnetts test p < 0.05. Cntrl is control. Cntrl is Control. Treatment dose means +/- 1 SE**

3.3. Discussion

In this experiment the oospore germination was not negatively impacted by any of the herbicide treatments or doses. This result has significant implications for the use of these herbicide products in our lakes and waterways for invasive weed management as it would mean that the herbicide products can be used with more confidence that they are not affecting native charophyte germination negatively. Although the results showed no negative impact on oospore germination, there were two treatments; endothall (1.25 ppm) and fluridone (0.1 ppm) which had a better germination count compared with the control (t-distribution test). The reason behind the better germination count in the treated jars is highly debatable and could just be part of the natural variation in oospore germination and a chance statistical event.

There were frequent examples of germination counts in treated jars being higher compared than the control depending on the species although the species-specific results were highly variable. *N. leonhardii* had a lower germination count compared with the control in the endothall, fluridone and mycoherbicide treatments. This apparent species sensitivity to herbicide treatments would require further investigations as the potential for herbicide impact on one species and not the others could have long-term implications for field application of herbicides. For example, the selection of one species over others could cause a potential loss of species diversity in the characean meadows as well as an impact on potential regeneration where a sensitive species dominates the seed bank.

There were two challenges with the mycoherbicide treatment. Firstly the mycoherbicide treatments developed algal growth which coated the sediment pots. The benthic algae growing in the mycoherbicide treatment jars may have induced lower oospore germination counts, as was shown in a study by Van den Berg *et al.* (2001) where *Chara* cf. *aspera* had low germination rates due to the sediment having high redox values caused by benthic algae oxygen production. Secondly, the mycoherbicide culture did not produce any spores when plated back, which indicates that the Mt isolate used was not virulent or pathogenic; meaningful conclusions cannot be drawn from the germination results. The spore count of the liquid culture used was only known several days after the liquid culture had been used, as the liquid cultures plated on water agar takes seven days to produce spores. The choice of liquid culture was based on the other viability tests (Section 2.2.5) which all indicated that the fungal isolate used was viable.

Chapter 4

4. Experiment 2: Sieved Oospore Germination

4.1. Introduction

The objective of this chapter is to examine whether the herbicide and mycoherbicide products negatively impact oospores germination when directly exposed to the herbicide treatments and whether sensitivity differs between charophyte species. The experimental hypothesis is that charophyte germination will not be affected by herbicide or mycoherbicide treatments at concentrations representing potential application rates.

This experiment involved placing aliquots of sieved mixed seed bank material (oospores and large debris) into containers and treating with three herbicides (diquat, endothall and fluridone), a mycoherbicide and untreated control. Each herbicide and mycoherbicide treatment had two dose concentrations.

4.2. Results

Mycoherbicide Culture

The liquid cultures used for inoculation had a microsclerotia count of 2.27 x 10^{-3}/mL, a biomass of 19.9 mg/mL and a zero spore count. The plated liquid cultures on PDA plates were free of contamination (Table 23-26, Appendix 2).

Experimental Harvests

Observations

Six days after treatment, the specialised vegetative propagules of *N.* aff. *cristata* started to germinate in some of the containers. By day 12 the debris in the containers treated with 2 ppm diquat was more yellow in colour compared to all the other treatments. By day 13 and 14 the mycoherbicide inoculum of the highest dose was slightly green in places and air bubbles trapped in the inoculum were causing some of the inoculum to lift off the sediment (Figure 32). By day 23 the mycoherbicide inoculum in the lowest dose starting to lift off the sediment surface due to trapped rising air bubbles.

Figure 32: **Mycoherbicide highest dose container 14 days after treatment. The mycoherbicide inoculum is floating on the surface (photograph by Author)**

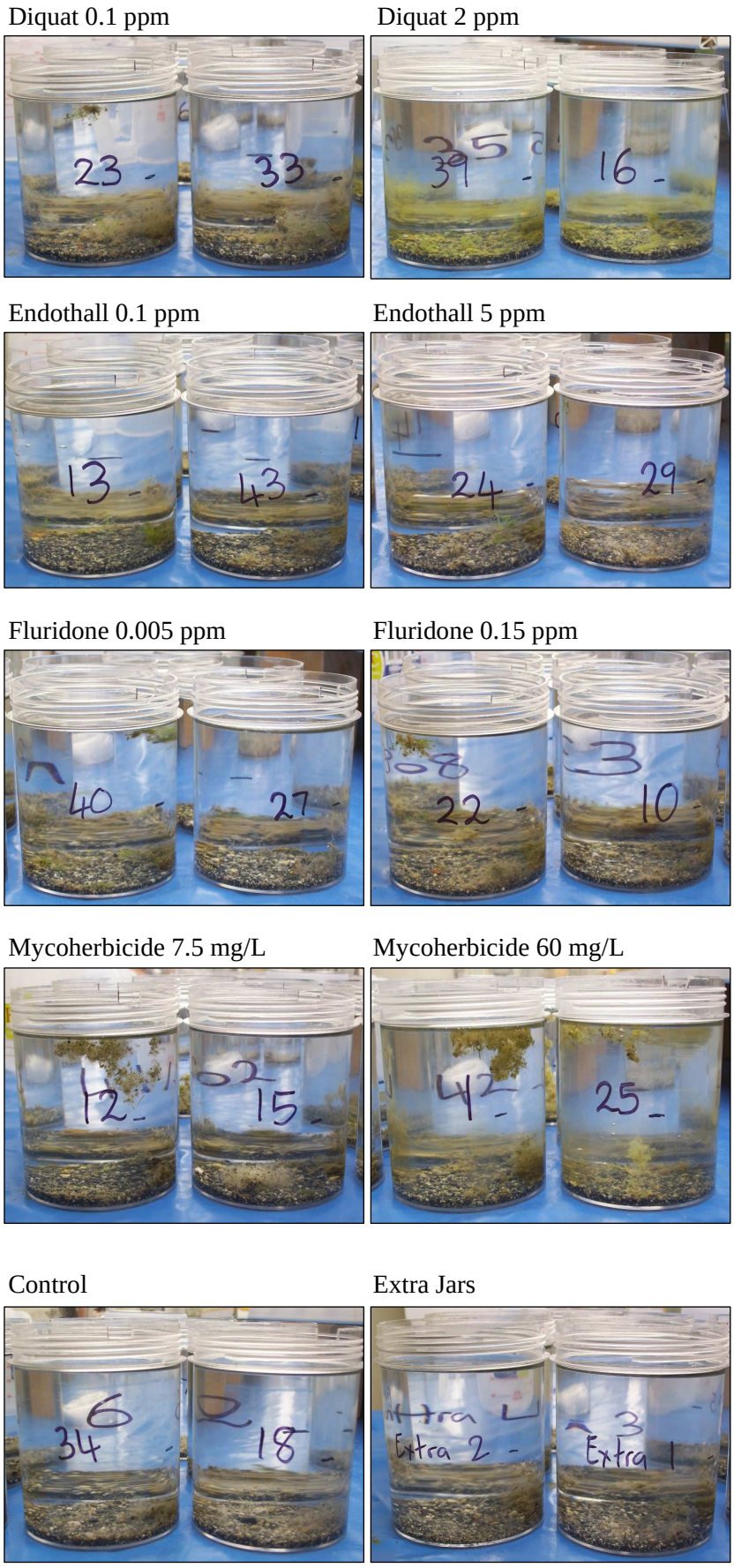


Figure 33: **Herbicide treatment doses, control and extra containers before harvest at 28 days after treatment (two of the five replicate containers) (photograph by Author).**

Germination and Species Count

There were no differences in either the germination or species count when the treatment doses were compared against the control (t-distribution test and Dunnett's tests) (Figure 34).

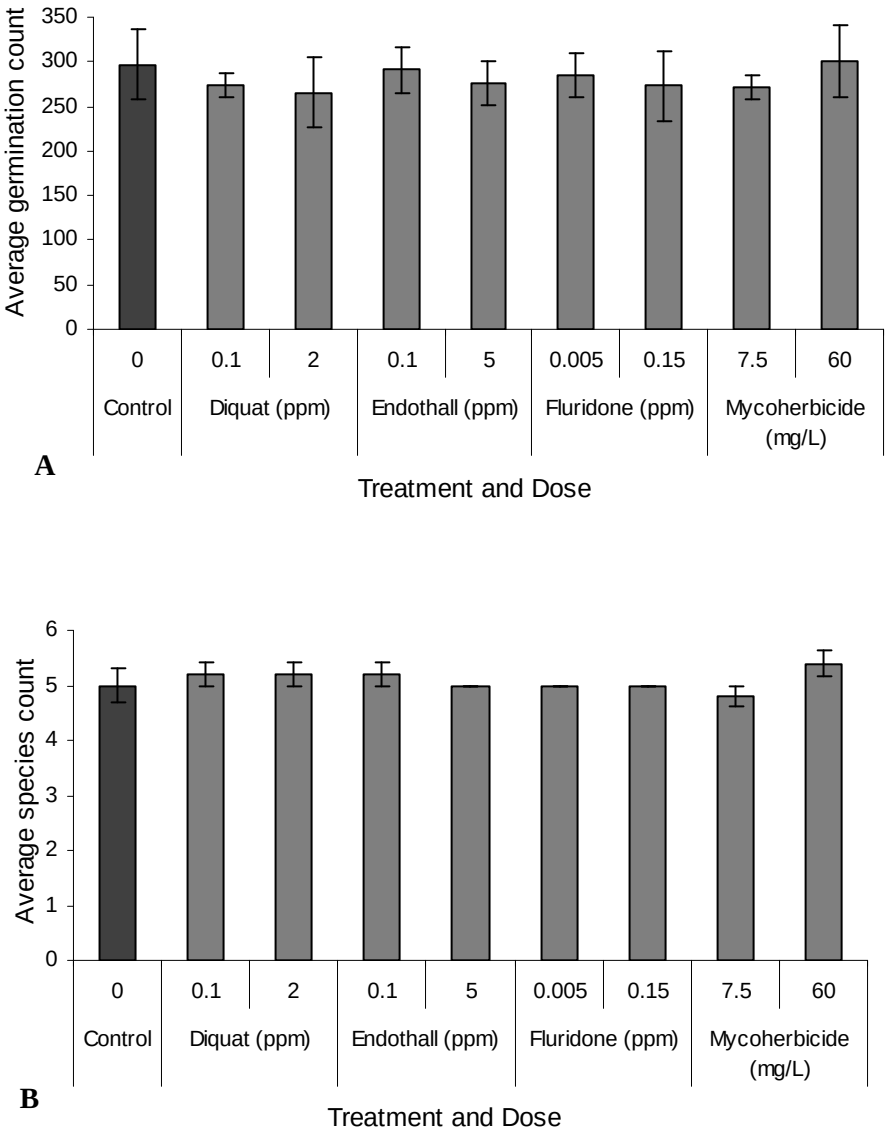


Figure 34: **Germination count (A) and species count (B) within all treatments. There were no statistically different results as indicated by t-distribution test and Dunnett's test. Treatment dose means +/- 1 SE**

Species Sensitivity

N. leonhardii

The germination count for all four treatments had a similar or higher germination count than the control containers (Figure 35). There were no statistical differences (t-distribution test) in the germination count when each treatment dose was compared with the control (Figure 35).

N.* aff. *cristata

The germination count for the mycoherbicide treatment was lower than the rest of the treatments and the control (Figure 35). In comparison with the control (t–distribution test), diquat at 0.1 ppm was the only treatment dose that had a significantly higher germination count than the control (Figure 35).

N. hyalina

The germination count for the mycoherbicide treatment was lower than the rest of the herbicides (excluding the control) (Figure 35). In comparison with the control (t-distribution test and Dunnett's test), there were two treatment doses, endothall at 0.1 ppm and fluridone at 0.15 ppm, which had a significantly higher germination count than the control (Figure 35).

N. pseudoflabellata

The germination count for all four treatments had a higher germination count than the control containers (Figure 36). When the treatment doses were compared with the control (t–distribution test) there were two treatment doses, diquat at 0.1 ppm

and mycoherbicide at 60 mg/L, which had a significantly higher germination count than the control (Figure 36).

C. globularis

The germination counts for diquat and fluridone treated containers were lower than the control or mycoherbicide treated containers (Figure 36). When the treatment doses were compared with the control (t–distribution test and Dunnett's test) there were three doses, diquat at 0.1, 2 ppm and fluridone at 0.15 ppm, which had significantly lower germination counts than the control (Figure 36).

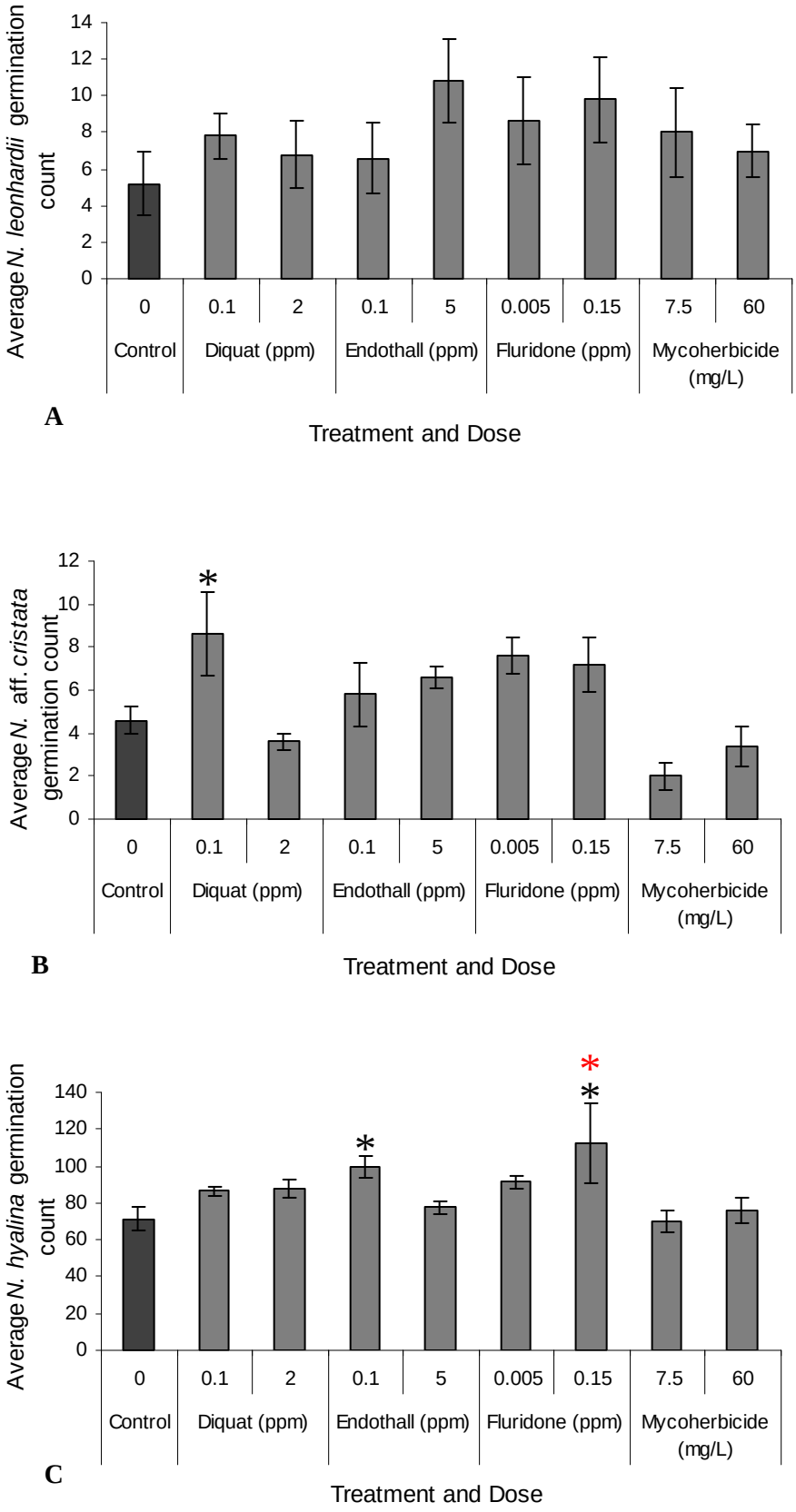


Figure 35: **Germination count within all treatments. (A) *N. leonhardii*, (B) *N.* aff. *cristata* and (C) *N. hyalina*. * = t-distribution test $p < 0.05$, * = Dunnett's test $p < 0.05$. Treatment dose means +/- 1 SE**

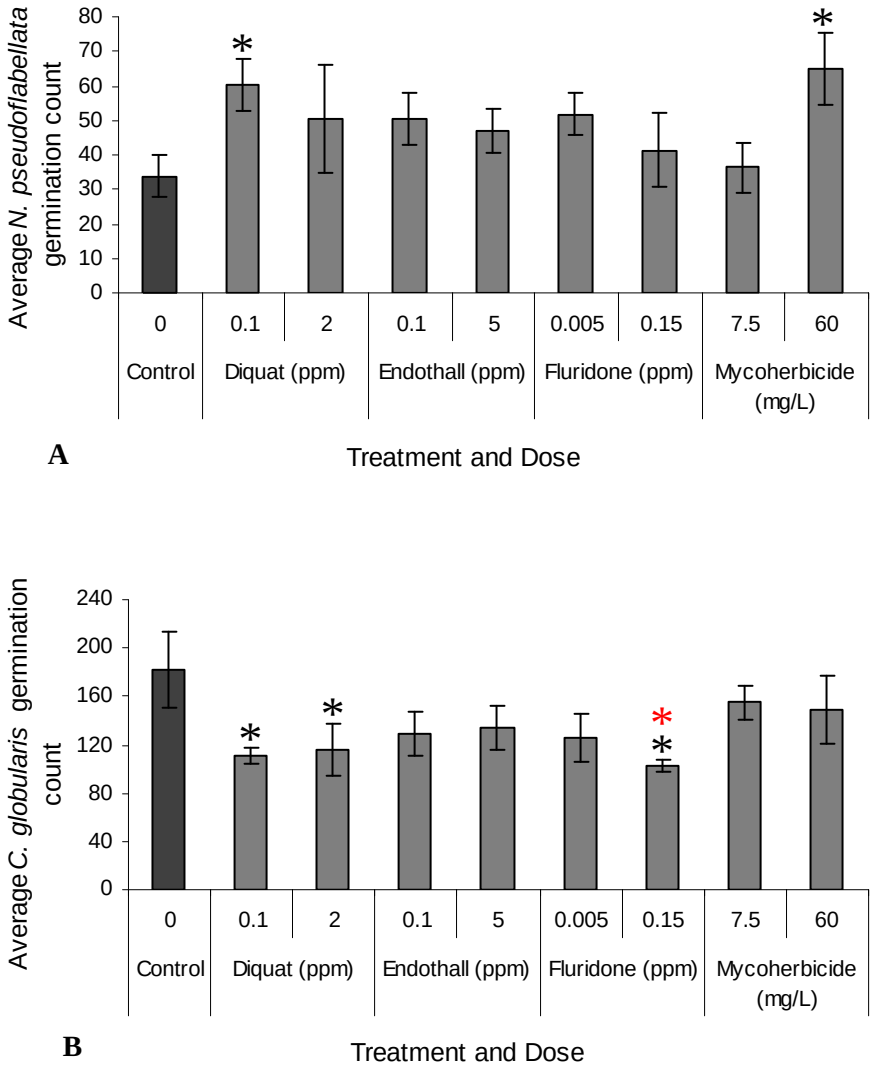


Figure 36: **Germination count within all treatments. (A) *N. pseudoflabellata* and (B) *C. globularis*. * = t-distribution test p < 0.05, * = Dunnett's test p < 0.05. Treatment dose means +/- 1 SE**

4.3. Discussion

In this experiment the oospores were directly exposed to constant herbicide dose concentrations. In addition, removing lake sediment would have reduced both the microbes that would potentially degrade the herbicides and the possibility of the diquat cation binding to negatively charged clay and sediment particles (Chapter 1.2.1), therefore increasing exposure of oospores to products.

The oospore germination and species count was not negatively impacted by any of the herbicide treatments or doses. Similarly when the data for each species was evaluated independently, there were frequent examples of germination counts in treated containers being higher compared with the control. However *C. globularis* had a lower germination count compared with the control in the diquat and fluridone treatments. This apparent species sensitivity would require further investigations as noted above for Experiment 1.

The lack of impact on the germination of the exposed oospores in the containers may be due to natural variation in oospore germination, and imparted resistance of dense oospore cell walls. Charophyte oospores have thick multilayered cell walls which are thought to make them resistant against desiccation and grazing (Casanova, 1991, 1997; Leitch, 1989), therefore, the resistant cell walls of oospores may also provide protection from herbicides. A fluridone experiment on charophyte oospore germination (Burkhart & Stross, 1990) showed that the oospore germination was unaffected by the fluridone treatment even at the maximum treatment dose (10 ppm). This result was similar to the results obtained in the present study.

As in the first experiment, there were two challenges with the mycoherbicide treatment. Firstly, the mycoherbicide treatments apparently promoted algal growth (discoloured) and trapped air bubbles in the inoculum, lifting it off the sediment. Secondly the mycoherbicide culture did not produce any spores when plated which indicates that the Mt isolate used was not virulent or pathogenic. This may preclude interpreting any meaningful conclusions from these results.

Chapter 5

5. Experiment 3: Outdoor Germling Response

5.1. Introduction

The objective of this chapter is to examine whether or not the herbicide and mycoherbicide products negatively impact charophyte germling growth. Features of germlings/young charophyte plants may make them more susceptible to herbicide effects than oospores. The aquatic herbicides used in New Zealand (diquat and endothall) and fluridone have modes of action which disrupt and inhibit photosynthesis which could have detrimental effects on young germlings as they are now reliant on their photosynthetic energy sources for survival rather than their starch reserves. The experimental hypothesis is that germling charophyte species are not susceptible to herbicide or mycoherbicide treatments.

This experiment involved placing germling pots into outdoor tanks and treating each with the maximum label rate according to the manufacturer's specifications for optimal dosage for each herbicide (diquat, endothall and fluridone), mycoherbicide and a control. The treatment dose concentration was applied as a post–emergent application. The experimental results were obtained from harvesting at two different time intervals; 5 weeks and 10 weeks.

5.2. Results

Mycoherbicide Cultures

The liquid cultures used for inoculation had a microsclerotia count of 0.13 x 10^{-3}/mL, a biomass of 12.75 mg/mL and a zero spore count. The plated liquid cultures on PDA plates were free of visible contamination (Table 27-30, Appendix 2).

Visual Observations

Diquat

For the first three weeks of the experiment there was no observable diquat effect on any of the germlings in the five treated tanks, no detectable germling height increase, and the water in the tanks remained clear. By week 4 there was noticeable algal growth on the wall of the tanks and the germlings were coated in algae (Figure 37). Between weeks 5 and 10 the germlings remained coated in algae and the water clarity declined. There was no noticeable germling height increase.

Endothall

For the first week of the experiment the water in the tanks was cloudy, although this cleared by week 2 when there was noticeable browning of sediment surface and some germlings were slightly brown in colour. By week 4 there were algae growing on the tank walls and some germlings were pale while others were growing in height (Figure 37). By week 7 noticeably more germlings were growing in the tanks, and this trend continued until harvest (week 10).

Fluridone

For the first two weeks of the experiment, the water in two of the five tanks was slightly cloudy. By week 4 there were algae growing on the tanks walls and on the water surface in one of the tanks (Figure 37). By week 5 the majority of the germlings were slightly coated in algae. It was only by week 7 that some germlings began to increase in height and by week 10 most germlings had increased in height.

Mycoherbicide

For the first week of the experiment the germling pots and the tank walls in all 5 tanks were coated in inoculum and the water was cloudy for the first week after treatment. By week 2 the water was only slightly cloudy but no germlings were visible in the pots due to the heavy coating of inoculum. Bioturbation by chironomids was observed in some of the pots. By week 3 the inoculum coating was turning brown, the water was clearing and 4 of the 5 tanks had mosquito larvae in them. By week 4 there were algae coating the tank walls and germlings were observed (Figure 37). Water clarity had declined in one of the tanks by week 6 and by week 8 the water clarity had declined in four out of five tanks.

Control

For the first week of the experiment the tanks water was clear and there was no noticeable change in the germlings. By week 2 there was noticeable browning of sediment surface and some germlings were slightly brown in colour. By week 4 there were algae growing on the tank walls and some germlings were beginning to

grow (Figure 37). Over the successive weeks more germlings were noticeably growing.

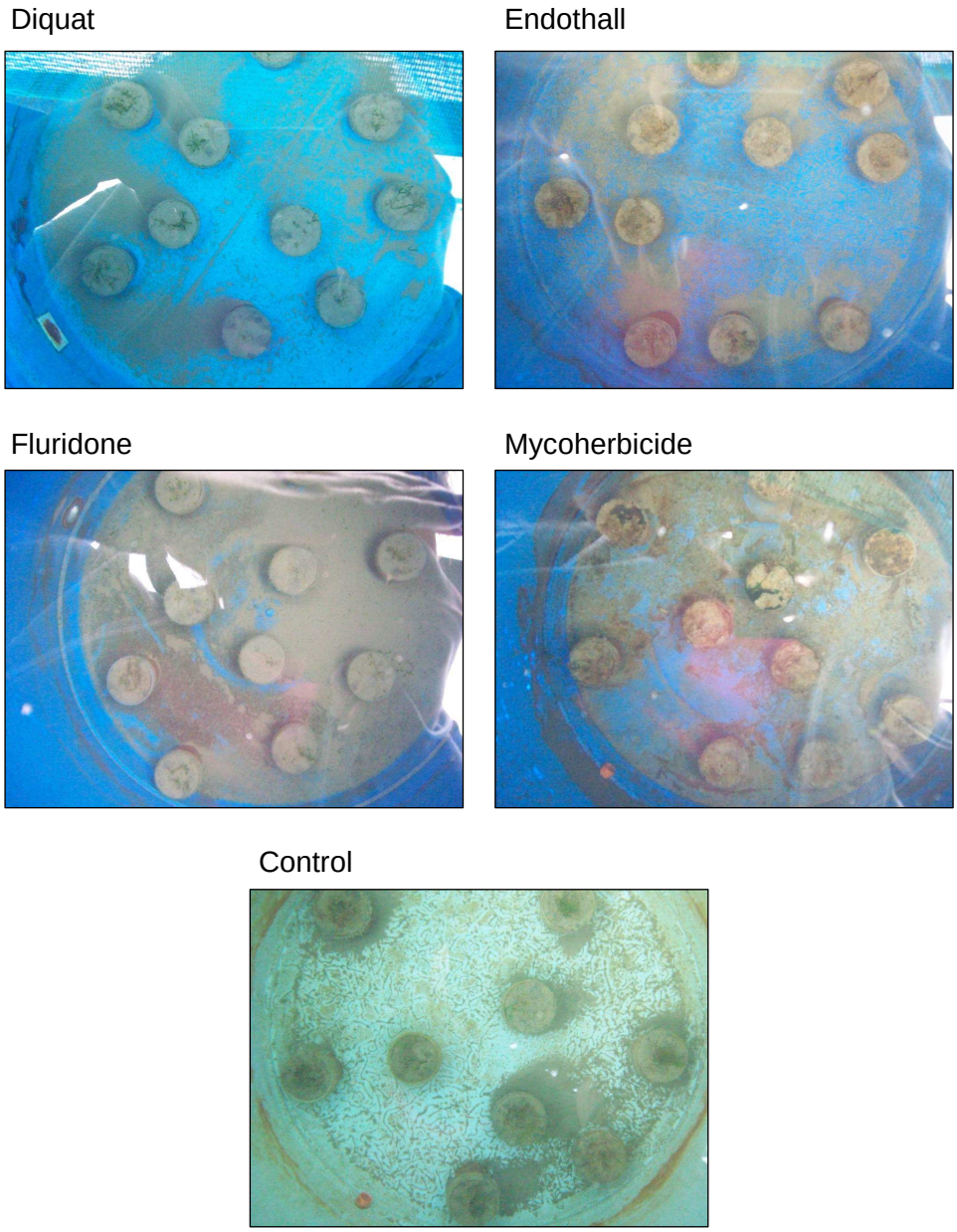


Figure 37: **Experimental tanks four weeks after treatment (one of the five replicate tanks) (photographs by Author)**

Biomass

In comparison to the initial pre–treatment germling biomass (23.96 mg) all treatments had decreased in biomass by the 5 week harvest. At the week 5 harvest,

the average charophyte biomass differed significantly ($p < 0.001$) between treatments with biomass in the diquat and mycoherbicide treated tanks, which were significantly lower than the control and endothall treated tanks (Tukey test). This is supported by Dunnett's test (Figure 38).

Ten weeks after treatment, only the control and endothall treatments had a higher biomass compared with the initial pre–treatment biomass. The average charophyte biomass differed significantly ($p < 0.05$) between treatments, with biomass from the diquat, fluridone and mycoherbicide treated tanks being lower than the control and endothall treated tanks. This was supported by the Dunnett's test, which showed two treatments, diquat and mycoherbicide, had significantly lower charophyte biomass than the control (Figure 38), but was not supported by the Tukey test.

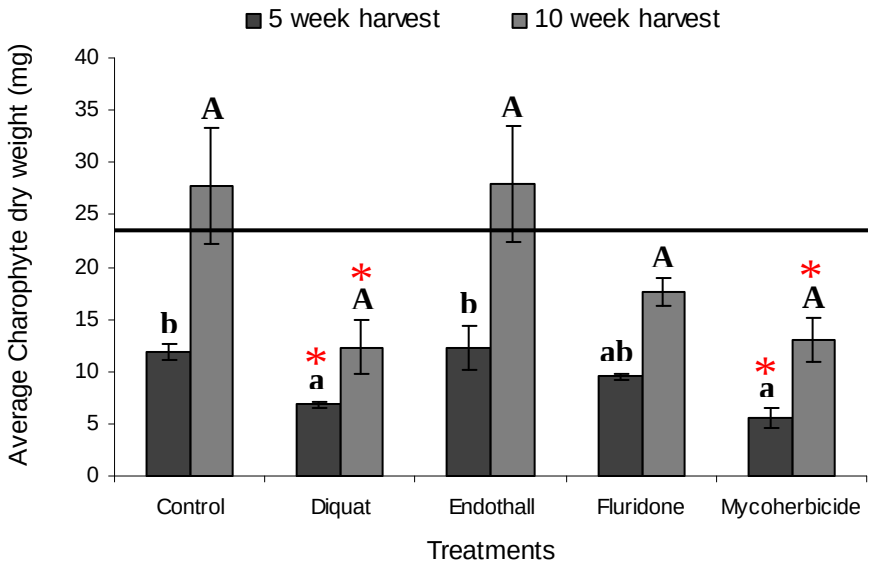


Figure 38: **Charophyte biomass recorded for the 5 and 10 week harvest. .Letters = Tukey test significance at 5% and * = Dunnett's test significant at 5%. Solid line is pre–treatment dry weight (23.96 mg). Treatment means +/- 1 SE**

5.3. Discussion

In Lake Wanaka during the 1970s, ‘double spraying’ of diquat was carried out to control *L. major* weed beds. Double spraying involved two successive herbicide applications in an attempt to control the re-growth that occurred on the *L. major* stems after the first spray (Clayton, 1986). This is one example of a treatment scenario that may result in exposing new charophyte germlings, (which have emerged from the sediment after the first herbicide application) to the herbicide. This present study examined germling susceptibility to herbicide treatments at the maximum label rate according to the manufacturer’s specifications for optimal dosage alongside untreated control germlings.

In this germling experiment (outdoor tanks), there were no sustained herbicidal effects on treated charophyte germlings after 10 weeks. This was despite two treatments (diquat and mycoherbicide) having lower germling biomass compared with the untreated control tanks, at five weeks after treatment. All the germlings had reduced biomass at the five week harvest compared with the pre–treatment biomass and treatment may have temporarily retarded germling growth.

Despite the use of a purified product comprising fungal mycelium, the mycoherbicide treatment appears to promote algal and/or bacterial development. Also, with no spores isolated in the viability tests, which indicates low virulence, these side-effects are likely to be driving the reduced germling performance with this treatment.

Several theories may explain the negative biomass results. One theory is that the initial shock of being transplanted from the pre–culture tank to the experimental tanks may have resulted in reduced biomass. Charophytes are delicate and easily damaged when handled due to large turgid cells which can snap and collapse (Coffey & Clayton, 1988). In a growth study by Casanova (1994) damage from handling during transplantation may have affected the growth rates of the charophytes when *C. australis* required 70 days of adjustment in the new environment while *N. sonderi* did not grow well and many individuals died. Another theory for reduced biomass is that some charophyte species may grow better under winter conditions than summer conditions. A final theory is that the coating of algae (epiphytic algae) on the germlings in the diquat-treated tanks may explain the low germling growth; however, this is only speculative. Epiphytic coating on plant leaves (which may deactivate any diquat present) has been shown to suppress macrophyte growth by interfering with carbon uptake, as well as affecting photosynthesis and reducing oxygen diffusion rates (Clayton & Champion, 2003). A specific example includes a study done on *Potamogeton perfoliatus* which demonstrated that *P. perfoliatus* heavily coated with epiphytic algae had significantly reduced chlorophyll a, above-ground biomass accumulation and net photosynthesis compared with *P. perfoliatus* without an epiphytic algal coating (Asaeda et al., 2004).

Chapter 6

6. Experiment 4: Germling Response to Herbicides and Algaecide

6.1. Introduction

The objective of this chapter is to examine whether or not the herbicide and mycoherbicide products negatively impact charophyte germling growth. The experimental hypothesis is that germling charophyte species are not susceptible to herbicide or mycoherbicide treatments.

The experimental work involved placing germling and *L. major* pots into glass jars with three herbicides (diquat, endothall and fluridone), a mycoherbicide, an algaecide (chelated copper compound) and an untreated control. The *L. major* jars were treated with one dose (maximum label rate according to the manufacturer's specifications for optimal dosage) while the charophyte treatment jars were treated with three doses for each for the herbicides, mycoherbicide and algaecide. All doses were applied as a post–emergent application. The experiment ended with harvesting of germling and *L. major* biomass after 2 weeks.

This germling experiment had two controls. One control used a chelated copper algaecide known to be efficacious against charophytes (Guha, 1995; Leslie, 1990) to ensure the charophyte germlings did not exhibit unrelated effects. The second control for the experiment used a known target weed species (*L. major*) to test if the treatment concentrations applied were herbicidal and that no unrelated effects occurred. Chelated copper compounds are plant cell toxicants that disrupt cell membranes (Netherland, 2009) and are known to kill invasive weeds especially if

combined with another herbicide, for example, endothall (Durborow et al., 2007). The response of the green shoots of the target weed *L. major* to diquat, endothall and fluridone, is known from several New Zealand studies where diquat and endothall have complete efficacy on the green shoots of *L. major* causing total collapse to occur (Clayton, 1986; Hofstra & Clayton, 2001b; Wells & Clayton, 1993) while fluridone bleaches only the new growing shoots on plant stems causing, some of them to turn purple while the rest of the plant generally remains green and healthy (Hofstra & Clayton, 2001a; Wells et al., 1986).

6.2. Results

Mycoherbicide Culture

The liquid cultures used for inoculation had a microsclerotia count of 1.1 x 10^{-3}/mL, a biomass of 29.77 mg/mL and a zero spore count. The plated liquid culture grew well and had no visible contamination (Table 31-34, Appendix 2).

Lagarosiphon major

Observational Data

Over the two-week period of the experiment there was no observable impact on the *L. major* shoot in the control and mycoherbicide treated jars as the shoots remained healthy and green. Four days after treatment, the *L. major* shoots treated with diquat and endothall turned brown and by day 12 most of the shoots were dead, although there were noticeably healthy charophytes growing in the pots with the dead *L. major* shoots. *L. major* shoots treated with fluridone remained healthy and green with pink tips developing after 12 days. Four days after treatment the *L. major* shoots treated with K-Tea showed darkening leaf colour while the stem remained a healthy light green colour. Over time the darkened leaves became flaccid (day 12) and limp (day 14) while the stems remained healthy and new side shoots began to grow (Figure 39).

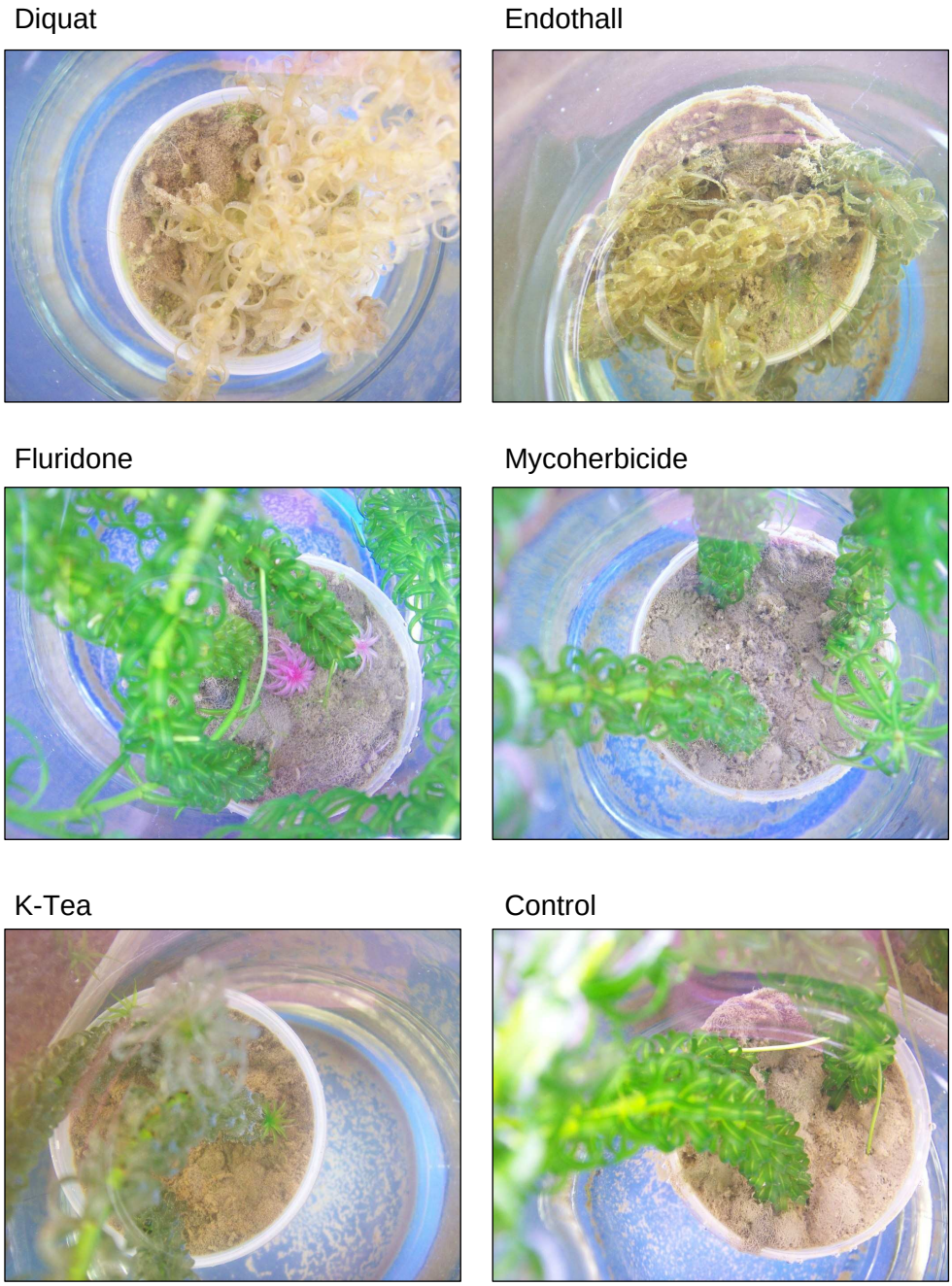


Figure 39: ***Lagarosiphon major*** **jars 14 days after treatment (one of five replicate jars shown) (photographs by Author)**

L. major Shoot Height Above Sediment

There was an increase in *L. major* shoot height above sediment level for the control, fluridone, mycoherbicide and chelated copper (K-Tea) treated jars. Only diquat and endothall decreased *L. major* shoot height and there were no recoverable shoots two weeks after treatment (Figure 40).

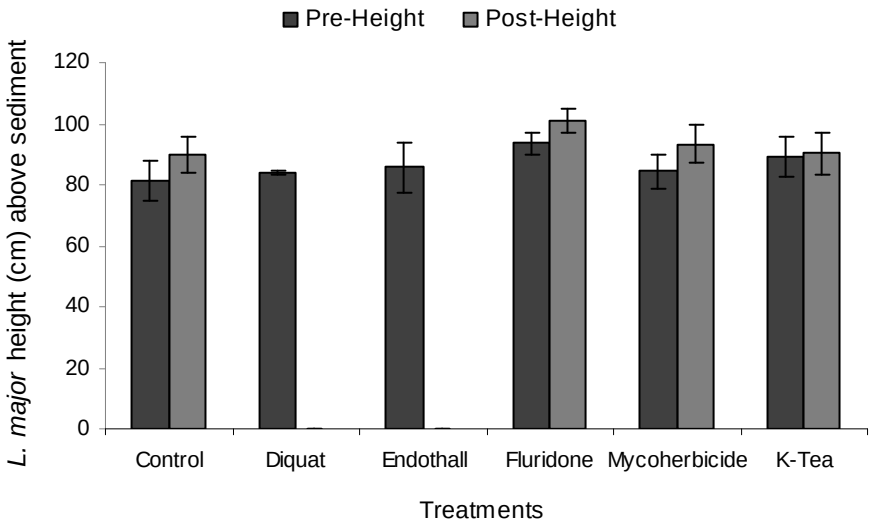


Figure 40: ***L. major*** **pre and post treatment height (cm) above sediment. Treatment means +/- 1 SE**

Biomass

In comparison to the initial pre–treatment biomass (0.62 g) only the control, fluridone and mycoherbicide treated jars showed increases in *L. major* biomass over the experimental timeframe (Figure 41). Diquat and endothall treatment achieved complete control, with no *L. major* biomass recovered from these treatments. In comparison with the control (t- distribution test) diquat, endothall and chelated copper treated jars had a significantly lower biomass while fluridone treated jars had a significantly higher biomass (Figure 41).

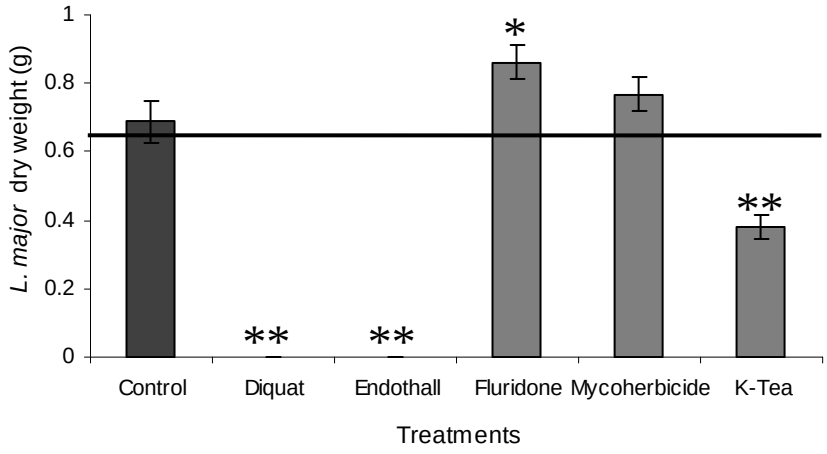


Figure 41: **Average *L. major* biomass (g) 14 days after treatment (DAT). Treatment means +/- 1 SE. * = t-distribution test significance at 5%, ** = t-distribution test significance at 1%. Solid line is pre-treatment dry weight (0.62 g)**

Charophyte

Observational Data

With the exception of the chelated copper treatment, most of the treated pots had germlings present throughout the two week experimental period with noticeable oospore germination and new germlings developing. The germlings treated with chelated copper were mostly brown, opaque and dead with only a few new germlings beginning to emerge through the sediment by the end of the experiment (Figure 42 and Figure 43).

Figure 42: **Control treatment 14 days after treatment (one of five replicates shown) (photograph by Author)**

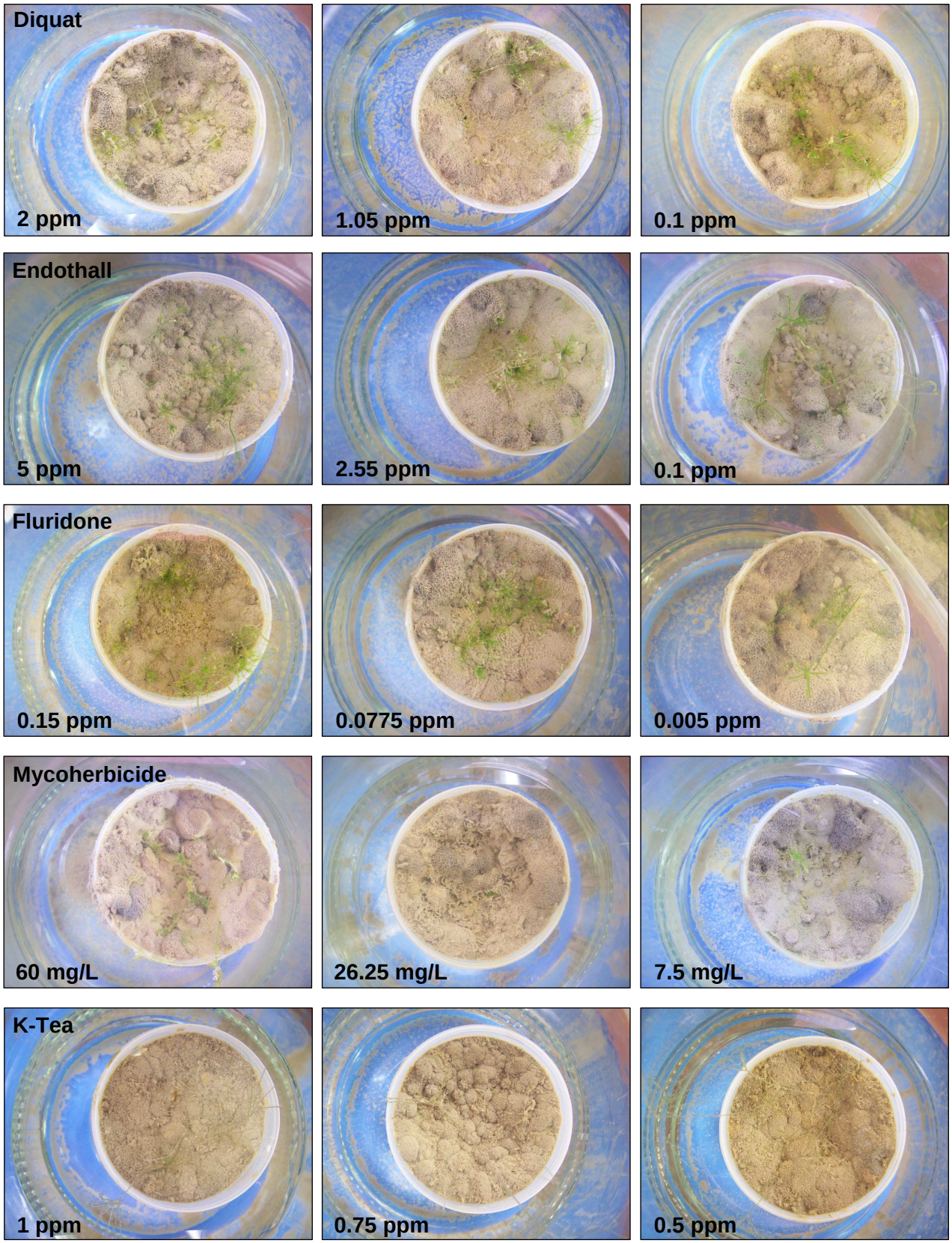


Figure 43: **Herbicide treatment dose 14 days after treatment (one of five replicates shown) (photographs by Author)**

Charophyte Height Above Sediment

Replicates showed highly variability of charophyte height (Figure 44) where most of the germlings measured at the end of the experiment were shorter in height (Figure 44).

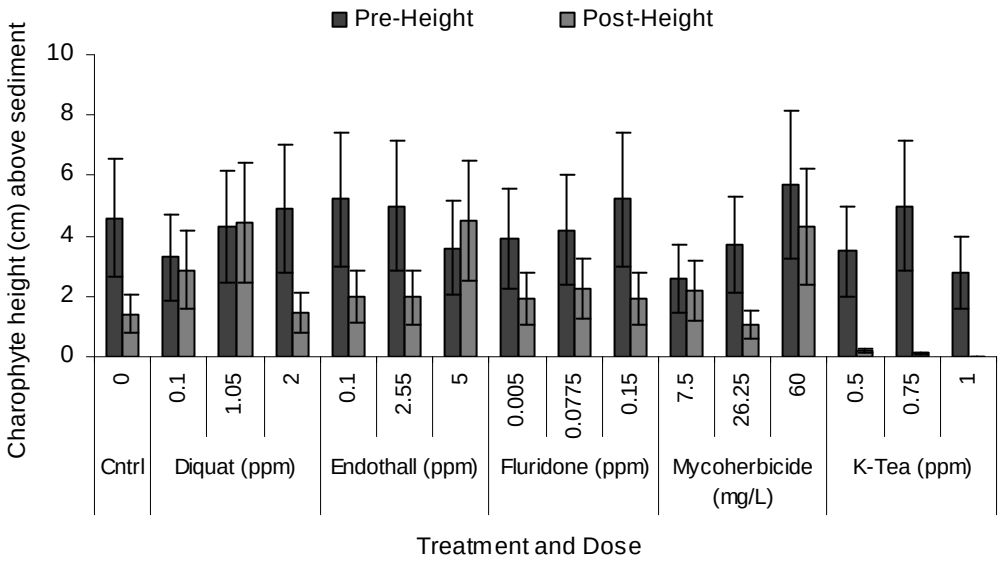


Figure 44: **Charophyte pre and post treatment height (cm) above sediment. There were no statistically different results as indicated by t-distribution test and Tukey test. Cntrl is control. Treatment dose means +/- 1 SE**

Biomass

The charophyte biomass was lower in the chelated copper treatments than all the other herbicides and the control (Figure 45). There were no significant differences ($p > 0.05$, Figure 45) in charophyte biomass within each treatment. Both endothall and mycoherbicide treatments had a higher charophyte biomass in the top dose compared to the other two doses while in the copper treatments charophyte biomass was negligible.

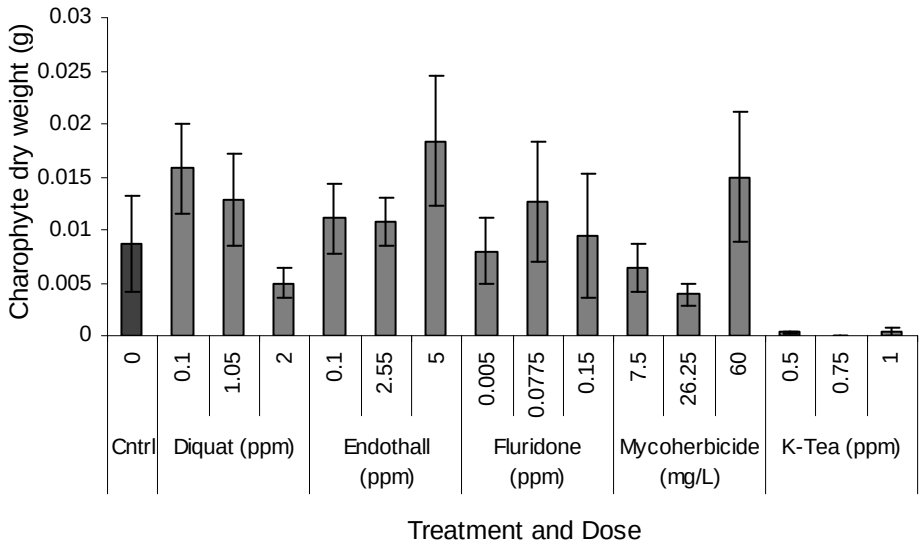


Figure 45: **Charophyte biomass 14 days after treatment. Cntrl is control. Treatment dose means +/- 1 SE**

6.3. Discussion

This germling experiment used treatments of different herbicides and concentrations alongside untreated control germlings, and two positive controls, a charophyte treatment control (chelated copper compound) for the germlings and a target plant control (*L. major*) for the herbicides.

The germlings in the charophyte control treatment (chelated copper) died as expected. Chelated copper is an algaecide and has been used routinely to control charophytes in other countries. This confirmed that charophytes were susceptible to herbicide effects under the experimental conditions used in this study. The *L. major* plants in the herbicide response control died (diquat and endothall) or had herbicidal symptoms (fluridone and copper) which were all indicative of known herbicide effects on *L. major* plants, especially with the fluridone treatment since fluridone is a slow-acting herbicide (45+ days) and the length of the experiment (ca. 2 weeks) may have limited the potential of its effect on *L. major* biomass. The *L. major* plants treated with mycoherbicide showed no sign of infection which is a likely due to cultures not producing spores, indicating that the Mt isolate used was not virulent or pathogenic. Presence of Mt was confirmed, however, by plates prepared from treated *L. major* (Appendix 3).

The charophyte germling biomass results indicated that only one treatment (chelated copper, K-Tea) negatively impacted the germling biomass. The charophyte height above sediment (excluding chelated copper treatment) indicated that the germlings were probably slightly affected in this experiment (decrease in height), but the effects were masked by regeneration of new plants through

germination of oospores still in the seed bank. This result has significant implications for the use of these herbicides products in lakes and waterways for invasive weed management because if successive herbicide applications are carried out, germling establishment is still likely to be supported by continual germination from the sediment. However, the decreased germling height may be a result of handling the germling pots when measuring heights and transferring the pots from the water containers on the floor to the glass jars on the table.

The charophyte biomass differences within the treatments in this germling experiment could be part of the natural variation in germling growth. For example, chance timing of germination may drive the differences in the composition of germlings, such as, the plants with earliest germination could gain a competitive advantage (Casanova & Brock, 1990; de Winton et al., 2000). Nevertheless, the variable results within the treatments were not significantly different from the control.

Chapter 7

7. Conclusion

7.1. Summary

The results of the research presented in this thesis indicate that oospore germination was not negatively impacted by the type of herbicide used at any of the potential field application rates. Species sensitivity was evident in these results, however, the cause or mechanism for this is not currently understood. It is possible that there was sensitivity to the herbicides or a combination of naturally occurring factors. The potential for species sensitivity to herbicide use in lakes remains unclear. However, if herbicide use impacts one species and not the others this could have long term implications for field applications, such as the selection of one species over others. This could cause a dominance of certain more resistant charophyte species, resulting in the localised loss of species diversity in the characean meadows as well as seed banks. Of note though is the localised nature of any such response, given the relatively short contact times of herbicide use.

Similarly, the germling growth in all treatments over the five to ten week period (outdoor experiment) has positive implications for field applications of herbicides as it indicated no sustained herbicidal effects on treated charophyte germlings. Only the diquat treatment showed a temporary negative impact on germlings biomass compared with the untreated control germlings.

Under controlled temperature and light conditions charophyte germlings were not negatively impacted by the type of herbicide (except chelated copper) used at any

of the potential application rates. Both of the controls (chelated copper on germlings and herbicides on *L. major*) indicated that the response of charophyte germlings and *L. major* to their respective herbicide treatments were as expected. Handling of the germlings may have contributed to the reduced germling height between the pre- and post-treatment; however, herbicidal effects cannot be ruled out.

There have been numerous field observations which have indicated that the aquatic herbicides used in the present study have been successful on target weeds and have shown little to no impact (i.e. injury symptoms or biomass reduction) on established charophyte meadows (Clayton, 1986; Clayton & Tanner, 1988; Hofstra & Clayton, 2001b; Leonard & Creenland, 1965; Starling et al., 1974; Wells et al., 1986). However, their impact on critical early life stages of charophyte species was untested. To date germling and oospore germination has received relatively little study therefore the present study sought to provide insight into what effects, if any, herbicide and mycoherbicide products have on charophytes germination, germling susceptibility and species response. In this study the oospore germination and germling response have positive implications for field application of herbicides as they indicate that the timing of herbicide applications to lakes and waterways for invasive aquatic plant management is not crucial for charophyte survival. This is because the younger growth stages (oospores and germlings) were unaffected, especially in the long term (germlings). For management implications given the same level of target weed control (i.e. the level of target weed control is not compromised by product choice), the use of one product over another does not confer any advantage or benefit to native charophyte regeneration.

7.2. Future Work

Several areas requiring further investigation were identified by this thesis research. One question is to elucidate species sensitivity, with respect to product choice. Specifically to determine reproducibility and/or causal mechanisms for this sensitivity.

An option for testing species sensitivity to herbicide with regards to germination would be a variation of Experiment 1, whereby oospores would be extracted from the sediment and separated out into their different species. The extracted oospores would then be replaced either directly back into sterile sediment or into mesh bags and then into sterile sediment. Each species would be placed in its own separate experimental container. The lake sediment would be sterilised by autoclaving to prevent any oospores and other propagules in the sediment from germinating. Therefore, only the extracted oospores placed in the sediment after sterilisation would germinate. Similarly the second germination experiment from this thesis research could be repeated but with the sieved oospores separated by species and placed into separate containers.

For the germling experiments, further research would involve a different experimental method which would minimize the handling of the germlings. In addition, to test effects on germlings without continual germination from the sediment, the sediment would require the oospores to be extracted, sediment sterilized and the oospores placed back directly into the sediment or into mesh bags and then into the sediment.

The method of extracting and identifying oospores, as well as testing their viability is time consuming and in some cases may not be feasible. Care has to be taken when handling oospores as too much handling damages them. The long timeframe for extracting, identifying and testing the viability of oospores may result in premature germination of the oospores extracted first. When testing species sensitivity only one or two species would be able to be experimented on in parallel.

The shorter timeframe (ca. 2 weeks) in the fourth experiment (germling experiment) may have influenced the fluridone results as fluridone is a slow-acting herbicide (45 days) and the full effects of the fluridone treatment may not have been seen. Therefore, this experiment may need to be repeated over a longer experimental timeframe.

Finally, in all the experiments there was an ongoing problem with the virulence and pathogenicity of the Mt isolate used, as the culture produced no spores. Therefore, no conclusions could be drawn from that data relative to the original hypothesis. It would be beneficial to repeat the experiments using an Mt isolate that was virulent and pathogenic.

In conclusion, the results of this thesis indicate that charophyte oospores are able to germinate from the sediment after being treated with herbicides although some species-specific sensitivity was found. Charophyte germlings were initially slightly susceptible to herbicide treatment or handling but this was short lived as the germlings recovered and continual oospore germination was observed from

the sediment. Further research into species-specific responses to different herbicide products is essential as species sensitivity could result in a loss of charophyte species diversity. The future research proposed here will expand current knowledge of the effects of herbicides on charophytes and the potential for use of a mycoherbicide in control of invasive aquatic plants in New Zealand.

References

Asaeda, T., Sultana, M., Manatunge, J., & Fujino, T. (2004). The effect of epiphytic algae on the growth and production of *Potamogeton perfoliatus* L. in two light conditions. *Environmental and Experimental Botany, 52*(3), 225-238.

Avery, J. L. (2003). Aquatic weed management. Herbicide safety, technology and application techniques. *SRAC Publication No. 3601*, 8. Retrieved from http://www.aces.edu/dept/fisheries/aquaculture/pdf/3601fs.pdf

Bills, G. F., & Polishook, J. D. (1992). A new species of *Mycoleptodiscus* from living foliage of *Chamaecyparis thyoides*. *Mycotaxon, 43*, 453-460.

Blindow, I. (1992). Decline of charophytes during eutrophication: comparison with angiosperms. *Freshwater Biology, 28*, 9-14.

Bold, H. C., & Wynne, M. J. (1978). *Introduction to the algae: structure and reproduction*: Prentice-Hall Inc, Englewood Cliffs, New Jersey.

Bonis, A., & Grillas, P. (2002). Deposition, germination and spatio-temporal patterns of charophyte propagule banks: a review. *Aquatic Botany, 72*(3-4), 235-248.

Brown, J. M. A. (1975). Ecology of Macrophytes. In V. H. Jolly & J. M. A. Brown (Eds.), *New Zealand Lakes* (pp. 244-262). Auckland: Auckland University Press.

Burkhart, C. A., & Stross, R. G. (1990). An aquatic bioassay of herbicide bleaching in the charophyte sporeling, *Nitella furcata*. *Journal of Aquatic Plant Management, 28*, 50-51.

Casanova, M. T. (1991). An SEM study of developmental variation in oospore wall ornamentation of three *Nitella* species (Charophyta) in Australia. *Phycologia, 30*(3), 237-242.

Casanova, M. T. (1994). Vegetative and reproductive responses of charophytes to water-level fluctuations in permanent and temporary wetlands in Australia. *Australian Journal of Marine and Freshwater Research, 45*, 1409-1419.

Casanova, M. T. (1997). Oospore variation in three species of *Chara* (Charales, Chlorophyta). *Phycologia, 36*(4), 274-280.

Casanova, M. T., & Brock, M. A. (1990). Charophyte germination and establishment from the seed bank of an Australian temporary lake. *Aquatic Botany, 36*, 247-254.

Champion, P. D., Clayton, J. S., & Rowe, J. (2002). Lake managers handbook: alien invaders (pp. 51): Ministry for the Environment Publication, Wellington, ME number 4444.

Clayton, J. S. (1986). Review of diquat use in New Zealand for submerged weed control. *Proceedings of the EWRS/AAB 7th symposium on Aquatic Weeds*, 73-79.

Clayton, J. S., & Champion, P. D. (2003). Rotorua Lakes: plants tell the tale. *Proceedings of the "Rotorua Lakes 2003" A public symposium on Practical Management for Good Lake Water Quality*.

Clayton, J. S., & Matheson, F. (2010). Optimising diquat use for submerged aquatic weed management. *Hydrobiologia, 656*, 159-165.

Clayton, J. S., & Tanner, C. C. (1988). Selective control of submerged aquatic plants to enhance recreational uses of water bodies. *Verhandlungen des Internationalen Verein Limnologie, 23*, 1518-1521.

Closs, G., Dean, T., Champion, P. D., & Hofstra, D. E. (2004). Chapter 27: Aquatic invaders and pest species in lakes. In J. Harding, P. Mosley, C. Pearson & B. Sorrell (Eds.), *Freshwaters of New Zealand* (pp. 1-14). Christchurch, New Zealand: The Caxton Press.

Coffey, B. T., & Clayton, J. S. (1988). *New Zealand waterplants. A guide to plants found in New Zealand freshwaters*: Ruakura Agricultural Centre.

Coops, H. (2002). Ecology of charophytes: an introduction. *Aquatic Botany, 72*(3-4), 205-208.

de Winton, M. D., Casanova, M. T., & Clayton, J. S. (2004). Charophyte germination and establishment under low irradiance. *Aquatic Botany, 79*(2), 175-187.

de Winton, M. D., Champion, P. D., Clayton, J. S., & Wells, R. D. S. (2009). Spread and status of seven submerged pest plants in New Zealand lakes. *New Zealand Journal of Marine and Freshwater Research, 43*(2), 547-561.

de Winton, M. D., & Clayton, J. S. (1996). The impact of invasive submerged weed species on seed banks in lake sediments. *Aquatic Botany, 53*(1-2), 31-45.

de Winton, M. D., Clayton, J. S., & Champion, P. D. (2000). Seedling emergence from seed banks of 15 New Zealand lakes with contrasting vegetation histories. *Aquatic Botany, 66*(3), 181-194.

de Winton, M. D., Dugdale, T. M., & Clayton, J. S. (2007). An identification key for oospores of the extant charophytes of New Zealand. *New Zealand Journal of Botany, 45*, 463-476.

de Winton, M. D., & Schwarz, A.-M. (2004). Chapter 24: Littoral algal and macrophyte communities. In J. Harding, P. Mosley, C. Pearson & B. Sorrell (Eds.), *Freshwaters of New Zealand* (pp. 1-14). Christchurch, New Zealand: The Caxton Press.

Doong, R. L., MacDonald, G. E., & Shilling, D. G. (1993). Effect of fluridone on chlorophyll, carotenoid and anthocyanin content of Hydrilla. *Journal of Aquatic. Plant Management, 31*, 55-59.

Duarte, C. M. (2000). Marine biodiversity and ecosystem services: an elusive link. *Journal of Experimental Marine Biology and Ecology, 250*(1-2), 117-131.

Dugdale, T. M., de Winton, M. D., & Clayton, J. S. (2001). Burial limits to the emergence of aquatic plant propagules. *New Zealand Journal of Marine and Freshwater Research, 35*(1), 147-153.

Durborow, R. M., Tucker, C. S., Gomelsky, B. I., Onders, R. J., & Mims, S. D. (2007). Aquatic weed control in ponds. 24. Retrieved from http://www.ksuaquaculture.org/Pubs.htm/Aquatic%20Weed%20Control%20in%20Ponds%207-3-07.pdf

Gerdemann, J. W. (1953). An Undescribed Fungus Causing a Root Rot of Red Clover and Other Leguminosae. *Mycologia, 45*(4), 548-554.

Glomski, L. A. M., Skogerboe, J. G., & Getsinger, K. D. (2005). Comparative efficacy of diquat for control of two members of the Hydrocharitaceae: Elodea and Hydrilla. *Journal of Aquatic Plant Management, 43*, 103-105.

Guha, P. (1995). Exploring ecological control of Chara. *Crop Protection, 14*(6), 527-528.

Haas, J. N. (1994). First identification key for charophyte oospores from central Europe. *European Journal of Phycology, 29*(4), 227-235.

Hofstra, D. E., & Champion, P. D. (2006a). Management options assessment report for *Hydrilla verticillata* (pp. 20): NIWA client report HAM2006-159 Prepared for Biosecurity New Zealand (MAF07204).

Hofstra, D. E., & Champion, P. D. (2006b). Management options assessment report for *Lagarosiphon major* (pp. 30): NIWA client report HAM2006-161 Prepared for Biosecurity New Zealand (MAF07204).

Hofstra, D. E., & Champion, P. D. (2006c). Organism consequence assessment *Ceratophyllum demersum* (pp. 21): NIWA client report HAM2006-058e Prepared for Biosecurity New Zealand (MAF06205).

Hofstra, D. E., & Champion, P. D. (2006d). Organism consequence assessment *Egeria densa* (pp. 19): NIWA client report HAM2006-058g Prepared for Biosecurity New Zealand (MAF06205).

Hofstra, D. E., & Champion, P. D. (2006e). Organism consequence assessment *Lagarosiphon major* (pp. 16): NIWA client report HAM2006-058h Prepared for Biosecurity New Zealand (MAF06205).

Hofstra, D. E., & Champion, P. D. (2008). Assessment of environmental effects for the use of Endothall in four hydrilla affected lakes in Hawke's Bay (pp. 50): NIWA client report HAM2008-086 Prepared for Biosecurity New Zealand (MAF08208).

Hofstra, D. E., & Clayton, J. S. (2001a). Control of dioecious New Zealand Hydrilla using fluridone in mesocosms. *Journal of Aquatic Plant Management, 39*, 125-128.

Hofstra, D. E., & Clayton, J. S. (2001b). Evaluation of selected herbicides for the control of exotic submerged weeds in New Zealand: I. The use of endothall, triclopyr and dichlobenil. *Journal of Aquatic Plant Management, 39*, 20-24.

Hofstra, D. E., Clayton, J. S., & Getsinger, K. D. (2001). Evaluation of selected herbicides for the control of exotic submerged weeds in New Zealand: II. The effects of turbidity on diquat and endothall efficacy. *Journal of Aquatic Plant Management, 39*, 25-27.

Hofstra, D. E., Edwards, T., Clayton, J. S., & Shearer, J. F. (2009). New record of fungus *Mycoleptodiscus terrestris* from New Zealand. *New Zealand Journal of Botany, 47*, 411-415.

Howard-Williams, C., Clayton, J. S., Coffey, B. T., & Johnstone, I. M. (1987). Macrophyte invasions. In A. B. Viner (Ed.), *Inland waters of New Zealand* (pp. 307-331). Wellington: DSIR Science Information Publishing Centre.

Jackson, M. A. (1997). Optimizing nutritional conditions for the liquid culture production of effective fungal biological control agents. *Journal of Industrial Microbiology & Biotechnology, 19*, 180-187.

Kalin, M., & Smith, M. P. (2007). Germination of *Chara vulgaris* and *Nitella flexilis* oospores: What are the relevant factors triggering germination? *Aquatic Botany, 87*(3), 235-241.

Kufel, L., & Kufel, I. (2002). Chara beds acting as nutrient sinks in shallow lakes - a review. *Aquatic Botany, 72*(3-4), 249-260.

Leitch, A. R. (1989). Formation and ultrastructure of a complex, multilayered wall around the oospore of *Chara* and *Lamprothamnium* (Characeae). *European Journal of Phycology, 24*(3), 229-236.

Leonard, W. F., & Creenland, A. R. (1965). Control of aquatic weeds. *Proceedings of the NZ Weed and Pest Control Conference, 18*, 95-104.

Leslie, A. J. (1990). Aquatic use of copper-based herbicides in Florida. 14. Retrieved from http://www.fwc.state.fl.us/docs/WildlifeHabitats/InvasivePlants_copper.pdf

Madsen, J. D. (2009a). Appendix D: Developing a lake management plan. In L. A. Gettys, W. T. Haller & M. Bellaud (Eds.), *Biological control of aquatic plants: a best management practices handbook* (pp. 167-172). Marietta GA. 210 pages: Aquatic Ecosystem Restoration Foundation.

Madsen, J. D. (2009b). Chapter 1: Impact of invasive aquatic plants on aquatic biology. In L. A. Gettys, W. T. Haller & M. Bellaud (Eds.), *Biological control of aquatic plants: a best management practices handbook*. Marietta GA. 210 pages: Aquatic Ecosystem Restoration Foundation.

Mason, R. (1975). The Macrophytes. In V. H. Jolly & J. M. A. Brown (Eds.), *New Zealand Lakes* (pp. 231-243). Auckland: Auckland University Press.

Morris, J. E. (2009). Managing Iowa fisheries - Aquatic plant management (pp. 10). Retrieved from http://www.extension.iastate.edu/Publications/PM1352J.pdf

Netherland, M. D. (2009). Chapter 11: Chemical control of aquatic weeds. In L. A. Gettys, W. T. Haller & M. Bellaud (Eds.), *Biological control of aquatic plants: a best management practices handbook* (pp. 65-78). Marietta GA. 210 pages: Aquatic Ecosystem Restoration Foundation.

Netherland, M. D., Getsinger, K. D., & Skogerboe, J. D. (1997). Mesocosm evaluation of the species-selective potential of fluridone. *Journal of Aquatic Plant Management, 35*, 41-50.

Ostazeski, S. A. (1967). An Undescribed Fungus Associated with a Root and Crown Rot of Birdsfoot Trefoil (*Lotus corniculatus*). *Mycologia, 59*(6), 970-975.

Peterson, D. E., & Lee, C. D. (2005). Aquatic plants and their control (pp. 16): Kansas State University.

Santos, M. J., Anderson, L. W., & Ustin, S. L. (2011). Effects of invasive species on plant communitites: an example using submersed aquatic plants at the regional scale. *Biological Invasions, 13*, 443-457.

Schwarz, A.-M., de Winton, M. D., & Hawes, I. (2002). Species-specific depth zonation in New Zealand charophytes as a function of light availability. *Aquatic Botany, 72*(3-4), 209-217.

Shearer, J. F. (1994). Field test of first commercial formulation of Mycoleptodiscus terrestris (Gerd.) Ostazeski as a Biocontrol for Eurasian Watermilfoil. Miscellaneous Paper A-94-4 (pp. 22): U.S. Army Engineer Waterways Experiment Sattion, Vicksburg, MS.

Shearer, J. F. (1996a). *Development of a fungal pathogen for biocontrol of the submersed aquatic macrophyte Hydrilla verticillata.* Paper presented at the IX International Symposium on Biological Control of Weeds (eds. J. Hoffmann and C. Moran), Capetown, South Africa.

Shearer, J. F. (1996b). Potential of a pathogen, *Mycoleptodiscus terrestris*, as a biocontrol agent for the management of *Myriophyllum spicatum* in Lake Guntersville Reservoir. Technical Report A-96-4 (pp. 20): U.S. Army Engineer Waterways Experiment Sattion, Vicksburg, MS.

Shearer, J. F. (1997). Endemic pathogen biocontrol research on submerged macrophytes: status report 1996. Technical Report A-97-3 (pp. 26): U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

Shearer, J. F. (1998). Biological control of hydrilla using an endemic fungal pathogen. *Journal of Aquatic Plant Management, 36*, 54-56.

Shearer, J. F. (2002). Effects of a new growth medium on *Mycoleptodiscus terrestris* (Gerd.) Ostazeski. ERDC TN-APCRP-BC-04 (pp. 7): U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

Shearer, J. F. (2007). Some observations concerning microsclerotia and spore production of *Mycoleptodiscus terrestris* in culture. *Mycologia, 99*(1), 88-90.

Shearer, J. F., & Jackson, M. A. (2003). Mycoherbicide compositions and methods of preparing and using the same. US Patent 6,569.807. Washington, DC, US Patent Office.

Shearer, J. F., & Jackson, M. A. (2006). Liquid culturing of microsclerotia of *Mycoleptodiscus terrestris*, a potential biological control agent for the management of hydrilla. *Biological Control, 38*, 298-306.

Siemering, G. S., Hayworth, J. D., & Greenfield, B. K. (2008). Assessment of potential aquatic herbicide impacts to California aquatic ecosystems. *Archives of Environmental Contamination and Toxicology, 55*, 415-431.

Simsiman, G. V., Daniel, T. C., & Chesters, G. (1976). Diquat and endothall: their fates in the environment. *Residue Rev.*(62), 131-174.

Smith, G. M. (1950). *The fresh-water algae of the United States (2nd Edn.)*: McGraw-Hill Book Company, New York.

Starling, M. B., Chapman, V. J., & Brown, J. M. A. (1974). A contribution to the biology of *Nitella hookeri* A. Br. in the Rotorua Lakes, New Zealand. I. Inorganic nutritional requirements. *Hydrobiologia, 45*(1), 91-113.

Sutton, B. C., & Hodges Jr, C. S. (1976). Eucalyptus microfungi: *Mycoleptodiscus* species and *Pseudotracyella gen. nov*. *Nova Hedwigia, 27*, 693-700.

Tanner, C. C., Clayton, J. S., & Coffey, B. T. (1990). Submerged-vegetation changes in Lake Rotoroa (Hamilton, New Zealand) related to herbicide treatment and invasion by *Egeria densa*. *New Zealand Journal of Marine and Freshwater Research, 24*, 45-57.

Van den Berg, M. S., Coops, H., Meijer, M.-L., Scheffer, M., & Simons, J. (1998). Clear water associated with a dense *Chara* vegetation in the shallow and turbid Lake Veluwemeer, The Netherlands. In E. Jeppesen, M. Sondergaard, M. Sondergaard & Christoffersen (Eds.), *The structuring role of submerged macrophytes in Lakes* (pp. 339-352). USA: Springer-Verlag, New York.

Van den Berg, M. S., Coops, H., & Simons, J. (1999). Propagule bank build up by charophytes and its significance for colonization of a shallow lake. In M. S.

van den Berg (Ed.), *Charophyte colonization in shallow lakes: processes, ecological effects and implications for lake management* (pp. 41-56). RIZA report 99.015: Thesis Vrije Universiteit Amsterdam.

Van den Berg, M. S., Coops, H., & Simons, J. (2001). Propagule bank buildup of *Chara aspera* and its significance for colonization of a shallow lake. *Hydrobiologia, 462*, 9-17.

van Donk, E., & van de Bund, W. J. (2002). Impact of submerged macrophytes including charophytes on phyto- and zooplankton communities: allelopathy versus other mechanisms. *Aquatic Botany, 72*(3-4), 261-274.

Wade, P. M. (1990). The colonisation of disturbed freshwater habitats by *Characeae*. *Folia Geobotanica & Phytotaxonomica 25*(3), 275-278.

Watanabe, T., Moya, J. d. D., Gonzalez, J. L., & Matsuda, A. (1997). *Mycoleptodiscus terrestris* from black pepper roots in the Dominican Republic. *Mycoscience, 38*, 91-94.

Wells, R. D. S., & Clayton, J. S. (1993). Evaluation of endothall for aquatic weed control in New Zealand. *Proceedings of the NZ Plant Protection Conference, 46*, 102-106.

Wells, R. D. S., Coffey, B. T., & Lauren, D. R. (1986). Evaluation of fluridone for weed control in New Zealand. *Journal of Aquatic Plant Management, 24*, 39-42.

Wells, R. D. S., de Winton, M. D., & Clayton, J. S. (1997). Successive macrophyte invasions within the submerged flora of Lake Tarawera, Central North Island, New Zealand. *New Zealand Journal of Marine and Freshwater Research, 31*(4), 449-459.

Wood, R. D., & Mason, R. (1977). Characeae of New Zealand. *New Zealand Journal of Botany, 15*, 87-180.

Appendix 1: Dilution Tables for Herbicide Treatment Doses

Table 16: Experiment 1: Dilution table for herbicide treatment doses based on concentration of active ingredient (AI)

Herbicide	Diluted Solution Concentration Ppm AI	Diluted Solution Volume mL	Concentrated Solution Concentration Ppm AI	Concentrated Solution Volume mL
Diquat	50	2 000	200 000	0.5
	2	10 000	50	400
	1.5	10 000	50	300
	1	10 000	50	200
	0.5	10 000	50	100
Endothall	50	4 030	403 000	0.5
	5	10 000	50	1 000
	3.75	10 000	50	750
	2.5	10 000	50	500
	1.25	10 000	50	250
Fluridone	50	4 170	417 000	0.5
	0.15	10 000	50	30
	0.1	10 000	50	20
	0.05	10 000	50	10
	0.005	10 000	50	1

Table 17: Experiment 2: Dilution table for herbicide treatment doses based on concentration of active ingredient (AI)

Herbicide	Diluted Solution Concentration Ppm AI	Diluted Solution Volume mL	Concentrated Solution Concentration Ppm AI	Concentrated Solution Volume mL
Diquat	50	2 000	200 000	0.5
	2	25 000	50	1 000
	0.1	25 000	50	50
Endothall	50	4 030	403 000	0.5
	5	25 000	50	2 500
	0.1	25 000	50	50
Fluridone	50	4 170	417 000	0.5
	0.15	25 000	50	75
	0.005	25 000	50	2.5

Table 18: Experiment 4: Dilution table for herbicide treatment doses based on concentration of active ingredient (AI)

Herbicide	Diluted Solution Concentration Ppm AI	Diluted Solution Volume mL	Concentrated Solution Concentration Ppm AI	Concentrated Solution Volume mL
Diquat	50	2 000	200 000	0.5
	2	20 000	50	800
	1.05	10 000	50	210
	0.1	10 000	50	20
Endothall	50	4 030	403 000	0.5
	5	20 000	50	2 000
	2.55	10 000	50	510
	0.1	10 000	50	20
Fluridone	50	4 170	417 000	0.5
	0.15	20 000	50	60
	0.0775	10 000	50	15.5
	0.005	10 000	50	1
K-Tea	50	3 200	80 000	2
	1	20 000	50	400
	0.75	10 000	50	150
	0.5	10 000	50	100

Appendix 2: Mycoherbicide Culture Evaluation Results

Experiment 1

Culture flask 3 was used for mycoherbicide inoculation in Experiment 1

Table 19: Mt microsclerotia counts at 10 fold dilution rate

Flask	50 µl sample	50 µl sample	Ave per 50 µl	MS $x10^{-3}$/mL
1	7	12	9.5	1.9
2	3	9	6	1.2
3	**11**	**16**	**13.5**	**2.7**
4	8	8	8	1.6
5	1	0	0.5	0.1
6	3	4	3.5	0.7

Table 20: Mt culture dry weights (wt) per mL of culture, three replicates per flask

Flask	Filter wt (mg)	Filter + Mt wt (mg)	Mt wt (mg/mL)	Average Mt wt (mg/mL)
1	26.7	41.3	14.6	
	27.1	39.5	12.4	
	26.5	41.3	14.8	13.9
2	27.1	40.7	13.6	
	27	40.7	13.7	
	26.8	40.2	13.4	13.6
3	**27.1**	**40.3**	**13.2**	
	26.9	**41.4**	**14.5**	
	26.3	**41.2**	**14.9**	**14.2**
4	27.2	38.5	11.3	
	27.4	37.8	10.4	
	26.6	36.6	10	10.6

Table 21: Spore counts from spore plates (WA plates) at the 10 fold dilution rate

Flask	Plate 1	Plate 2	Spores x 10^{-4}/mL
1	0	0	0
2	0	0	0
3	**0**	**0**	**0**
4	0	0	0

Table 22: Mt growth on PDA plates. Growth = yes (Y) / no (N)

Flask	Plate 1	Plate 2	Clean / Contaminated
1	Y	Y	Clean
2	Y	Y	Clean
3	**Y**	**Y**	**Clean**
4	Y	Y	Clean

Experiment 2

Culture flasks 1 - 3 were used for mycoherbicide inoculation in Experiment 2

Table 23: Mt microsclerotia counts at 10 fold dilution rate

Flask	50 µl sample	50 µl sample	Ave per 50 µl	MS x10^{-3}/mL
1	9	5	7	**1.4**
2	18	20	19	**3.8**
3	9	7	8	**1.6**
4	3	5	4	0.8
5	2	0	1	0.2
6	1	1	1	0.2

Average of flasks 1-3 (**bolded)** = 2.27 x 10^{-3}/mL

Table 24: Mt culture dry weights (wt) per mL of culture, three replicates per flask

Flask	Filter wt (mg)	Filter + Mt wt (mg)	Mt wt (mg/mL)	Average mg wt (mg/mL)
1	26.6	44.3	17.7	
	26.7	44.9	18.2	
	26.4	46.1	19.7	**18.53**
2	27	45.9	18.9	
	26.5	46.6	20.1	
	26.6	46.5	19.9	**19.63**
3	27.3	49.9	22.6	
	27.3	46.5	19.2	
	27.3	49.7	22.4	**21.40**

Average Mt weight per mL of culture = 19.9 mg/mL

Table 25: Spore counts from spore plates (WA plates) at the 10 fold dilution rate

Flask	Plate 1	Plate 2	Spores x 10^{-4}/mL
1	0	0	**0**
2	0	0	**0**
3	0	0	**0**

Table 26: Mt growth on PDA plates. Growth = yes (Y) / no (N)

Flask	Plate 1	Plate 2	Clean / Contaminated
1	Y	Y	Clean
2	Y	Y	Clean
3	Y	Y	Clean

Experiment 3

All culture flask were used for mycoherbicide inoculation in Experiment 3

Table 27: Mt microsclerotia counts at 10 fold dilution rate

Flask	50 µl sample	50 µl sample	Ave per 50 µl	MS $x10^{-3}$/mL
1	4	1	2.5	0.5
2	2	0	1	0.2
3	1	0	0.5	0.1
4	0	1	0.5	0.1
5	0	0	0	0
6	0	1	0.5	0.1
7	0	0	0	0
8	1	2	1.5	0.3
9	0	0	0	0
10	0	1	0.5	0.1
11	1	0	0.5	0.1
12	2	2	2	0.4
13	0	0	0	0
14	1	0	0.5	0.1
15	0	0	0	0

Average of all flasks = 0.13 x 10^{-3}/mL

Table 28: Mt culture dry weights (wt) per mL of culture, one sample per flask

Flask	Filter wt (mg)	Filter + Mt wt (mg)	Mt wt (mg/mL)
1	27.4	46	18.6
2	28.3	34.2	5.9
3	27.7	35.6	7.9
4	28.2	44.3	16.1
5	28.3	43.9	15.6
6	27.8	39.9	12.1
7	27.9	41.6	13.7
8	27.1	31.6	4.5
9	27.3	39.8	12.5
10	27.1	41.6	14.5
11	27.1	39.5	12.4
12	27.1	44.2	17.1
13	27.5	41.6	14.1
14	27.1	44.1	17
15	27.5	36.8	9.3

Average Mt weight per mL of culture = 12.8 mg/mL

Table 29: Spore count from spore plates (WA plates) at the 10 fold dilution rate

Flask	Plate 1	Plate 2	Spores x 10^{-4}/mL
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	0	0	0
6	0	0	0
7	0	0	0
8	0	0	0
9	0	0	0
10	0	0	0
11	0	0	0
12	0	0	0
13	0	0	0
14	0	0	0
15	0	0	0

Table 30: Mt growth on PDA plates. Growth = yes (Y) / no (N)

Flask	Plate 1	Clean / Contaminated
1	Y	Clean
2	Y	Clean
3	Y	Clean
4	Y	Clean
5	Y	Clean
6	Y	Clean
7	Y	Clean
8	Y	Clean
9	Y	Clean
10	Y	Clean
11	Y	Clean
12	Y	Clean
13	Y	Clean
14	Y	Clean
15	Y	Clean

Experiment 4

Culture flasks 1 and 4 were used for mycoherbicide inoculation in Experiment 4 based on microsclerotia count and dry weights of the liquid cultures.

Table 31: Mt microsclerotia counts at 10 fold dilution rate

Flask	50 µl sample	50 µl sample	Ave per 50 µl	MS $x10^{-3}$/mL
1	5	7	6	**1.2**
2	4	4	4	0.8
3	2	1	1.5	0.3
4	5	5	5	**1**
5	0	1	0.5	0.1
6	2	3	2.5	0.5

Average of flasks 1 and 4 (**bolded)** = 1.1 x 10^{-3}/mL

Table 32: **Mt culture dry weights (wt) per mL of culture. Three replicates for the first two flasks and one sample for the remaining flasks**

Flask	Filter wt (mg)	Filter + Mt wt (mg)	Mt wt (mg/mL)
1	27.3	55.7	**28.4**
	27	54.6	**27.6**
	26.3	53.2	**26.9**
2	26.4	59.1	32.7
	26.6	58.5	31.9
	27.2	59.2	32
3	27.1	60.5	33.4
4	27.5	63.7	**36.2**
5	27.2	53.2	26
6	27.8	65.5	37.7

Average of flasks 1 and 4 (**bolded)** = 29.77 mg/mL

Table 33: **Spore counts from spore plates (WA plates) at the 10 fold dilution rate**

Flask	Plate 1	Plate 2	Spores x 10^{-4}/mL
1	0	0	0
2	0	0	0

NB. Flask 4 had no spore counts done

Table 34: **Mt growth on PDA plates. Growth = yes (Y) / no (N)**

Flask	Plate 1	Plate 2	Clean / Contaminated
1	Y	Y	Clean
2	Y	Y	Clean

NB. Flask 4 had no growth plates done

Appendix 3: Re-isolating Mt from Mycoherbicide Treated *L. major* Jars

Rose Bengal Agar (RBA) plates were inoculated with water from each of the five treated mycoherbicide *L. Major* jars, where each jar had three replicate plates (Table 35, Figure 46). There were four positive results on the RBA plates where seven Mt colonies grew.

Table 35: Water from the mycoherbicide treated *L. major* jars plated onto RBA plates (three plates per 2L jar) (x = no Mt)

L. major jar	Plate 1	Plate 2	Plate 3
1	x	x	x
2	x	x	x
3	x	x	x
4	x	3 putative Mt colonies.	1 putative Mt colony
5	2 putative Mt colonies	1 putative Mt colony	x

Leaves of Mt-treated *L. major* were plated onto PDA to test for Mt on the leaves. There was confirmation that Mt was present on the leaves when Mt was isolated back from most of the plated leaves. There was only one treatment jar which had no Mt growth from the plated leaves (Table 36, Figure 46).

Table 36: ***L. major*** **leaves, from 5 mycoherbicide-treated** ***L. major*** **jars, plated onto PDA plates (3 plates per 2L jar) (x = no Mt, #/# leaves = No. of leaves with Mt present/No. of leaves plated)**

Plate 1	Plate 2	Plate 3
3/3 leaves	3/3 leaves	3/3 leaves
4/4 leaves	3/3 leaves	2/3 leaves
x	1/3 leaves	1/4 leaves
2/3 leaves	3/3 leaves	3/3 leaves
x	x	x

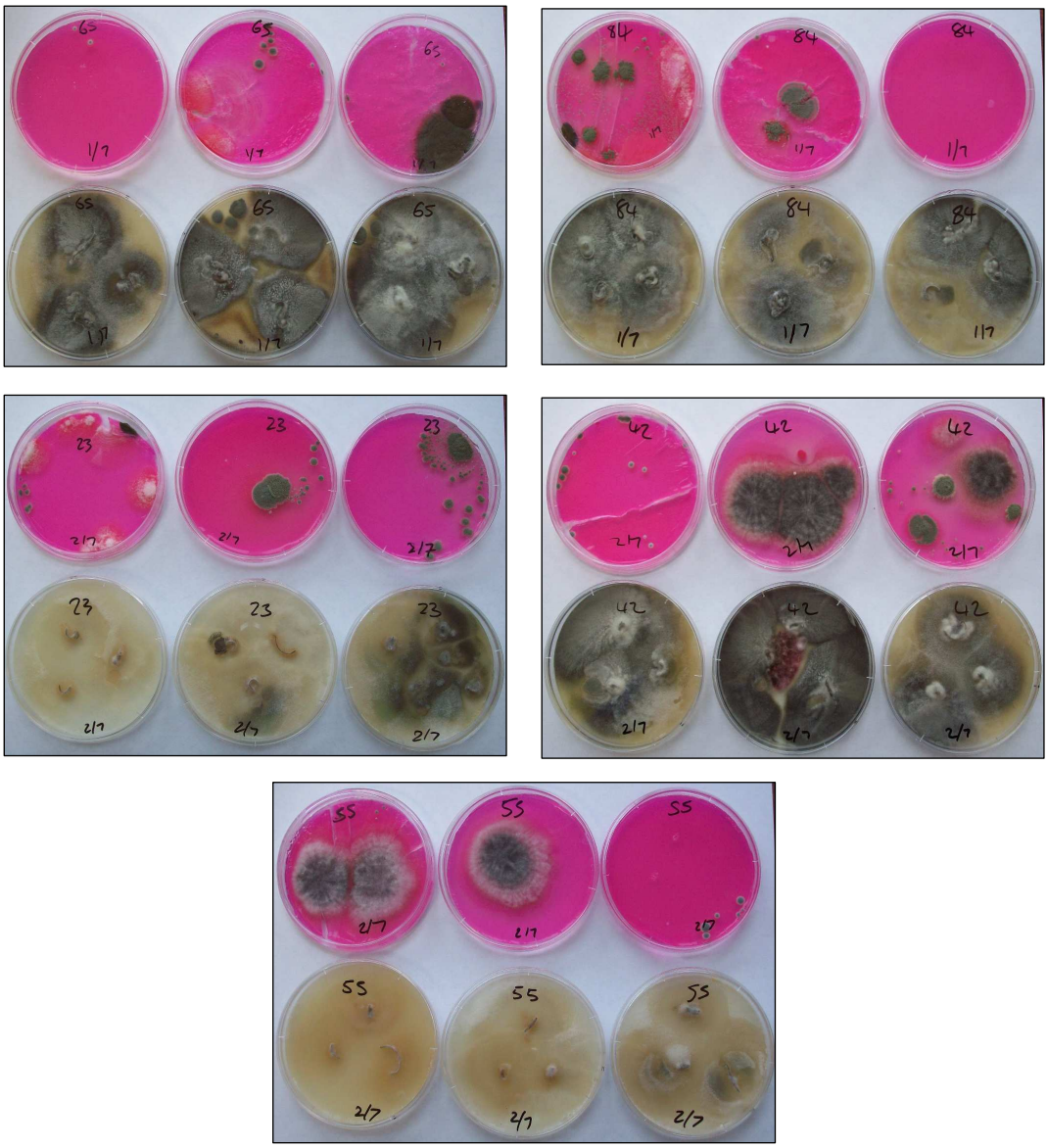


Figure 46: **Plating water and leaves from** ***L. major*** **jars treated with mycoherbicide. Pink plates are RBA plates and creamy yellow plates are PDA plates. Each picture shows three replicate RBA and PDA plates per jar treated with mycoherbicide (photographs by Author)**